

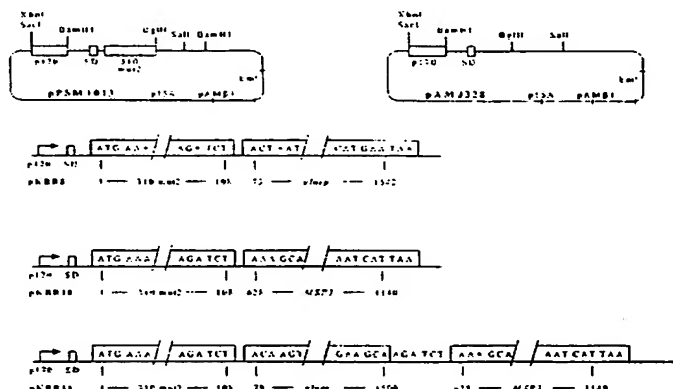


PCT

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000 1001 1002 1003 1004 1005 1006 1007 1008 1009 1010 1011 1012 1013 1014 1015 1016 1017 1018 1019 1020 1021 1022 1023 1024 1025 1026 1027 1028 1029 1030 1031 1032 1033 1034 1035 1036 1037 1038 1039 1040 1

- Published:**
— *with international search report*

(54) Title: MALARIA VACCINE



WO 2004/043488 A1

BEST AVAILABLE COPY



— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Malaria vaccine

Field of invention

5

An antigen based vaccine against malaria comprising fusion proteins derived from *Plasmodium falciparum* Glutamate-rich protein (GLURP) genetically coupled to at least one other *Plasmodium falciparum* derived protein, e.g. the Merozoite surface protein 3 (MSP3), or a vaccine comprising the DNA encoding this fusion protein and the production of such a vaccine.

10

Background

15

Malaria is affecting 40% of the world's population with an estimated 1.5 -2.7 million deaths annually (57). This represents a tremendous human suffering and a burden that prevents the development of the affected endemic communities. Malaria is now almost confined to the poorest tropical areas of Africa, Asia and Latin America, but transmission is being reintroduced to areas where it had previously been eradicated.

20

Malaria is one of the world's greatest public health problems.

The increasing emerging of insecticide resistant vectors and drug resistant parasites calls for investment in new and better control tools. Malaria vaccines hold the potential to dramatically alleviate the burden of malaria. However, our understanding of the mechanisms underlying protective immunity is incomplete hence specific markers of protection still needs to be defined.

25

An effective malaria vaccine will require the induction of appropriate humoral and cellular immune responses, against several key parasite antigens expressed during the various stages of the parasite life cycle. Each stage in the life cycle provides an opportunity for a vaccine.

30

Two lines of evidence suggest that a malaria vaccine is attainable:
Firstly, it is a well-established observation that repeated exposure to malaria parasites can lead to the development of solid clinical immunity, a status of premunition with concomitant existence of parasites and protective antibodies. Clinically immune

35

individuals generally have a lower parasite density and the immunity is quite effective at reducing mortality.

Secondly, experiments in humans as well as in animal models have established that immunizations can induce immunity against subsequent challenge with parasites suggesting that vaccination can become a realistic tool for malaria control.

In now classical experiments, Cohen and colleagues demonstrated that the passive transfer of antibodies purified from clinically immune individuals could ameliorate acute malaria attacks in African children with life-threatening *P. falciparum* infections (10). Druilhe and coworkers confirmed Cohen's results (42). They showed that IgG from clinically malaria immune West Africans were able - in a strain-independent manner - to substantially decrease the parasite load in asymptomatic Thai children with drug resistant *P. falciparum* malaria.

These groundbreaking passive transfer experiments have proven that antibodies are crucial in reducing / eliminating the asexual stage parasite load.

However, *in vitro* investigations with the same "protective" IgG preparations (42) demonstrated that antibodies do not inhibit parasite growth on their own, but act synergistically with blood mononuclear cells to control parasite multiplication (5). This parasite containing mechanism is referred to as antibody-dependent cellular inhibition (ADCI) (5, 26, 31). Recent studies have further demonstrated that binding of cytophilic antibodies such as IgG1 and IgG3 in conjunction with blood mononuclear cells via their FcγIIa receptors trigger the release of killing factors such as tumor necrosis factor-α (6).

Immuno-epidemiological studies support the *in vivo* relevance of a monocyte-dependent, antibody-mediated mechanism by showing a correlation between the acquisition of clinical immunity and levels of IgG1 and IgG3 antibodies, which bind well to the monocyte FcγRIIa receptor (1, 41). The putative involvement of this receptor in the development of immunity against clinical malaria is also supported by the finding that allelic polymorphism in FcγRIIa is associated with differential susceptibility to *P. falciparum* malaria (45). Kenyan infants homozygous for the FcγRIIa-Arg131 allele are reported to be less at risk from high-density *P. falciparum* infections compared with children with the heterozygous Arg/His131 genotype (45). Since the FcγIIa-Arg131 genotype (but not the FcγIIa-His131 genotype) binds strongly to IgG1 and IgG3, this finding supports the notion that monocyte-mediated killing of *P. falciparum* is an

important mechanism for parasite containment *in vivo*. Additionally, Aucan et al (2) found that levels of specific IgG2 antibodies - but not IgG3 and IgG1 - were associated with protection from clinical malaria in a population from Bukina Faso. Subsequent sequencing of FcγRIIa revealed that 70% of the study subjects had the FcγRIIa-H131 allele. This
5 allele binds strongly to IgG2 (56), suggesting that IgG2 is acting as a cytophilic subclass in this population (2). Collectively these observations suggest that the FcγRIIa genotype is an important factor for the development of immunity to clinical malaria and lends support to the validity of *in vitro* ADCI model.

10 The development of a vaccine for malaria has become increasingly recognized as a high priority in the effort to control malaria worldwide due to the increasing incidence of drug-resistant disease. New tools are therefore required to facilitate the clinical evaluation of candidate vaccines, particular the validation of *in vitro* correlates of the protection afforded by vaccination. ADCI may provide one such tool (13). The currently
15 most prominent blood-stage vaccine candidates MSP1, MSP2, AMA1, and RESA have primarily been selected for clinical testing because of their ability to induce growth-inhibitory antibodies in pre-clinical animal models (9, 16, 16, 30, 55). However, despite initial promises, they have in general proved poorly immunogenic in the human volunteers (18, 25, 29, 43) and the induced antibodies were unable to inhibit the *in vitro*
20 growth of *P. falciparum*. Thus, the *in vitro* invasion inhibition assay is not ready to serve as a surrogate marker of immunity.

The lack of suitable correlates of human protection that reflect inhibition of merozoite invasion has encouraged the development of other *in vitro* models that reflect possible
25 killing mechanisms in clinically immune individuals. Druilhe and coworkers have hypothesized that antibodies act synergistically with human blood monocytes to control parasite growth *in vivo* and have accordingly developed the *in vitro* correlate of this killing mechanism - the ADCI assay. We have so far identified two antigens - GLURP and MSP3 - that are targets of ADCI-effective human antibodies.

30 The *Plasmodium falciparum* Glutamate-rich protein (GLURP) and the Merozoite surface protein 3 (MSP3) are both targeted by human IgG antibodies, which can inhibit parasite growth *in vitro* in a monocyte-dependent manner (36, 52) and *in vivo* in the humanized SCID mouse model (3). The similar effects of human antibodies against
35 these antigens are also suggested by a number of immuno-epidemiological studies, which demonstrate that the levels of GLURP and MSP3 specific cytophilic antibodies

(IgG1 and IgG3) are significantly associated with a reduced risk of malaria attacks (11, 38, 50).

5 The discovery of GLURP and MSP3 is based on the *in vitro* analysis of passive transfer of immunity by purified African Immunoglobulin G (5, 6, 14, 42). These investigations have led to the elucidation of a putative effector mechanism in the defense against *P. falciparum* malaria (12), and the subsequent identification of the involved parasite molecules. The major B-cell epitopes recognized by these human IgG antibodies have been localized to conserved sequences in the GLURP₂₇₋₄₈₉ and MSP3₂₁₂₋₂₅₇ regions, 10 respectively (36, 50, 51). These studies lead to the identification of the N-terminal region of GLURP (GLURP₂₇₋₄₈₉) (52) and the C-terminal region of MSP3, (MSP3₂₁₀₋₃₈₀) (36) as targets of biologically active antibodies.

15 Different regions of these antigens have previously been produced in *Escherichia coli* fused to various affinity-tags (35, 53, 54). Whereas such additional sequences are advantageous for purification they also pose a potential problem because host immune responses against such sequences may render them useless for repeated applications.

Immune epidemiological investigations confirmed the relevance of anti-GLURP and anti-MSP3 IgG antibodies to acquired protection:
20 For GLURP, three independent studies performed in Dielmo, Senegal (38), Dodowa, Ghana (11, 50) and OoDo, Myanmar (Soe Soe, unpublished) have demonstrated a statistically significant correlation between levels of GLURP-specific IgG3 and/or IgG1 antibodies and protection against malaria attack. This association was highly significant even after controlling for the confounding effect of age-related exposure to *P. falciparum*. These results confirm previous studies, which found that naturally occurring 25 IgG antibodies to GLURP are associated with protection against disease in Gambian children (15) and against high levels of parasitemia in children from Liberia (21) and Burkina Faso (4).

For MSP3, a high ratio (> 2) of cytophilic to non-cytophilic antibodies (IgG1 + IgG3 / 30 IgG2+IgG4+IgM) allowed to distinguish individuals without recorded malaria attacks from individuals with malaria attacks. This was found in every age group among approximately 200 villagers from Dielmo who have been under daily clinical surveillance for more than 8 years (37). At the individual level, the occurrence of anti-MSP3 IgG3 antibodies was strongly associated with protection, in contrast to antibodies

of other isotypes directed against the same molecule or antibodies of any isotype directed against 5 other antigens (37).

A similar consistency in seroepidemiological data is not common for any other malaria vaccine candidate as exemplified by MSP1, the hitherto leading candidate as a vaccine against *P. falciparum* malaria.

The major B-cell epitopes recognized by these human IgG antibodies have been localized to conserved sequences in the GLURP₂₇₋₄₈₉ and MSP3₂₁₂₋₂₅₇ regions, respectively (36, 50, 51). These studies lead to the identification of the N-terminal region of GLURP (GLURP₂₇₋₄₈₉) (52) and the C-terminal region of MSP3, (MSP3₂₁₀₋₃₈₀) (36) as targets of biologically active antibodies.

Sequence analyses of the GLURP₂₇₋₄₈₉ and MSP3₂₁₀₋₃₈₀ regions from 44 field isolates and laboratory lines of *P. falciparum* show that defined epitopes in GLURP (P1, P3, and P4) (48) and MSP3 (b peptide) (34), which are targeted by ADCI-effective human antibodies are almost completely conserved, suggesting that they are functionally constrained and not subject to selection for variation at the amino acid level. Of the different epitopes in the GLURP₂₇₋₄₈₉ region, P3 might be the most important, since affinity-purified human antibodies against the P3 peptide mediated the strongest ADCI-effect *in vitro* (51). The conservation of major B-cell epitopes in GLURP and MSP3 is further supported by the observation that they are almost identical between *P. falciparum* and the closely related parasite *Plasmodium reichenowi*; a natural parasite for Chimpanzees (39, 53), and that plasma IgG antibodies from 71 adult Liberians clinically immune to malaria display identical binding patterns towards recombinant proteins representing the GLURP₂₇₋₅₀₀ regions from both species (53).

Collectively, these findings demonstrate that GLURP and MSP3 B-cell epitopes recognized by biologically effective human antibodies are conserved between geographically distant *P. falciparum* isolates and functionally constrained, suggesting that a vaccine based on GLURP and MSP3 may protect against a broad range of parasite strains worldwide.

In vitro experiments showed that naturally occurring affinity-purified human antibodies to GLURP (52) and MSP3 (36) could inhibit parasite growth in a monocyte-dependent

manner, whereas control antibodies affinity-purified on 7 other malarial vaccine candidates were unable to exert a similar effect (47).

The same inhibitory effect was obtained using naturally occurring affinity-purified IgG antibodies against both recombinant proteins (GLURP₂₇₋₄₈₉, and GLURP₇₀₅₋₁₁₇₈) (52) and synthetic peptides derived from the GLURP R0 region, P3 (GLURP₉₃₋₂₀₇), S3 (GLURP₄₀₇₋₄₃₄), and LR67 (GLURP₈₅₋₃₁₂) (50, 51), respectively.

In vivo experiments where affinity-purified MSP3b-specific human antibodies were passively transferred into *P. falciparum* infected Hu-RBC BXN mice, showed a parasite clearance as fast as that induced by Chloroquine, and faster than that induced by total African IgG (3). The latter observation indicates that immunization with selected antigens may lead to stronger immunity than that induced by the whole parasite (3).

In vivo experiments where *Aotus* monkeys immunized with recombinant MSP3 in Freund's complete adjuvant were fully protected against an experimental *P. falciparum* challenge (20). Immunizations of *Saimiri sciureus* monkeys have demonstrated that GLURP₂₇₋₅₀₀ adsorbed to Al(OH)₃ is non-toxic, immunogenic and elicit high titers of anti-GLURP antibodies which recognize *P. falciparum* by IFA (8). In a subsequent challenge with *P. falciparum* infected erythrocytes, two out of three monkeys were partially protected, this effect being directly related to the titer and epitope specificity of the antibodies developed by the primates in response to the immunogen (8).

These findings strongly support the notion that immune responses against GLURP and MSP3 B-cell epitopes that elicit ADCC-effective antibodies controls parasite multiplication *in vivo*.

Different regions of these antigens have previously been produced in *Escherichia coli* fused to various affinity-tags (35, 53, 54). Whereas such additional sequences are advantageous for purification they also pose a potential problem because host immune responses against such sequences may render them useless for repeated applications. It is therefore desirable to explore expression systems, which aims to produce the recombinant protein without a vector-encoded affinity-tag.

A restricted number of formulations based on MSP3 and GLURP have been select for further vaccine development and studied at the pre-clinical level first in mice (49, 54) and then in non-human primates challenged with *P. falciparum* (8). The N-terminal region of GLURP and the C-terminal region of MSP3 proved strongly immunogenic in pre-clinical models. These have now been produced individually using a new, highly efficient, expression system based on the pH and growth phase regulated promoter, P170, from *Lactococcus lactis* (23, 33).

We have so far identified two antigens - GLURP and MSP3 - that are targets of ADCI-effective human antibodies and recently performed two clinical phase I trials with the individual antigens. Both vaccines induced strong cellular responses in the volunteers, whereas the IgG antibody responses were moderate. All volunteers from the GLURP trial generated antibodies against the P3 B-cell epitopes, which is the most prominent target of ADCI-effective antibodies in clinically immune individuals. The relatively low levels of vaccine-induced antibodies may be related to the limited number of B-cell epitopes on the GLURP synthetic peptides.

It is therefore, desirable to develop a vaccine based on a recombinant protein, which include GLURP and MSP3 preferably with neighboring sequences containing additional B- and T-cell epitopes or other antigens from *P. falciparum* such as the CS-antigen. It is also desirable to use expression systems, which produces the recombinant protein without a vector-encoded affinity-tag, such as *L. lactis*.

Summary of the invention

A vaccine against malaria, which has an improved vaccine-induced antibody expression, is disclosed. The vaccine comprises a fusion protein derived from *Plasmodium falciparum* Glutamate-rich protein (GLURP) genetically coupled to at least one other *Plasmodium falciparum* derived protein, e.g. the Merozoite surface protein 3 (MSP3), or the corresponding nucleotide sequence coding said fusion protein.

Detailed disclosure of the invention

The present invention discloses an antigen based vaccine against malaria comprising a fusion protein derived from *Plasmodium falciparum* Glutamate-rich protein (GLURP)

genetically coupled to at least one other *Plasmodium falciparum* derived protein or homologues hereof.

5 A preferred embodiment of the invention is a vaccine where the protein genetically coupled to GLURP is derived from the Merozoite surface protein 3 (MSP3) from *Plasmodium falciparum* said fusion protein preferably having the amino acid sequence shown in SEQ ID NO 1.

10 In another embodiment the vaccine comprises SEQ ID NO 1 and further immunogenic epitopes of a protein derived from *Plasmodium falciparum*.

Also disclosed is the fusion protein as such with the amino acid sequence shown in SEQ ID NO. 1 and a fusion protein further comprising one or more immunogenic epitopes of one or more proteins derived from *Plasmodium falciparum*, such as CS, MSP1, MSP2, 15 MSP4, MSP5, MSP6, AMA1, Pf155/RESA, RAP1, EBA-175, pfEMP1, EXP1, LSA1, LSA3, Pf25, Pf45/48, Pf230, Pf27, Pf16, or Pf28 is suggested.

20 The present invention also regards the preparation of above mentioned fusion protein from a recombinant bacteria, e.g. *Lactococcus*.

In another aspect, the invention relates a nucleic acid encoding the above mentioned fusion protein and the use of said nucleic acid for preparing a vaccine. A preferred embodiment of a nucleic acid used for a vaccine is the sequence as shown in SEQ ID NO 2.

25 In still another embodiment the vaccine comprises a recombinant BCG containing a nucleic acid sequence encoding above mentioned fusion protein.

30 Since vaccines based on GLURP and MSP3 induce the same type of immune responses i.e. high levels of cytophilic antibodies and possibly complement each other as targets for the immunesystem, the respective GLURP₂₅₋₅₀₀ and MSP3₂₁₂₋₃₈₂ regions were introduced together as a recombinant hybrid in *Lactococcus lactis* in a novel gene expression system, which is based on the pH and growth phase regulated promoter, P170, from *L. lactis* (7, 23, 33, 56). This gene expression system offers a simple fermentation 35 procedure, which has been developed specifically for the P170 promoter. *L. lactis* was chosen as expression host because i) it is a well characterized industrial generally

recognized as safe (GRAS) microorganism, best known for its use in the production of fermented dairy products, ii) it can be grown in a defined synthetic medium, iii) recombinant proteins may be secreted into the culture supernatant, from where they can be easily purified, iv) it does not produce toxic substances.

5

The N-terminal region of GLURP and the C-terminal region of MSP3 have now been produced in a chimeric fusion protein, as a hybrid protein, using *L. lactis*.

10 The immunogenicity of the hybrid protein has been studied in mice with Montanide (Seppic) used as the adjuvant. Montanide was used in recent clinical trials with long synthetic peptides derived from GLURP and MSP3, respectively. Immunizations with the hybrid protein consistently generated a stronger antibody response against the individual GLURP and MSP3 domains than a mixture of the two molecules.

15

The difference was most pronounced for the MSP3-specific antibody response suggesting that T cell epitopes located in the GLURP R0-region provide help for B-cell epitopes in the MSP3 region. This is a surprising ability of the GLURP antigen which can be used with other malarial antigens also.

20

In contrast, when the animals were injected with a mixture of GLURP and MSP3, individual mice tended to mount a predominant antibody response against either molecule. In some animals GLURP was immune dominant whereas in other animals MSP3 was the dominant immunogen.

25

The hybrid was also more effectively recognized by naturally occurring IgG antibodies in clinically immune African adults than the individual antigens.

30 The GLURP-MSP3 hybrid protein therefore has four major advantages compared to the individual GLURP and MSP3 molecules:

- i) it is more immunogenic than any combination of the individual molecules,
 - ii) it generates a strong immune response against both GLURP and MSP3,
 - iii) it allows testing of both GLURP and MSP3 in a single clinical trial,
 - iv) it is predicted to be as safe as the individual molecules, since pre-clinical testing
- 35 in mice and in non-human primates has shown that it does not contain neo-epitopes in the fusion junction between GLURP and MSP3.

Other identified antigens from *P.falciparum* suitable as a fusion partner to the GLURP antigen are CS, MSP1, MSP2, MSP4, MSP5, MSP6, AMA1, Pf155/RESA, RAP1, EBA-175, pfEMP1, EXP1, LSA1, LSA3, Pf25, Pf45/48, Pf230, Pf27, Pf16, or Pf28.

5

Definitions

Fusion proteins

10 A recombinant fusion protein is encoded by a nucleotide sequence, which is obtained by genetically joining nucleotide sequences derived from different regions of one gene and/or by joining nucleotide sequences derived from two or more separate genes. These nucleotide sequences may be derived from *P. falciparum*, but they may also be derived from other organisms, the plasmids used for the cloning procedures or from other
15 nucleotide sequences.

Immunogenic fragment or epitope

An immunogenic fragment or epitope is defined as a part of the protein that induces an immune response in a biological sample or an individual currently or previously
20 infected with a microorganism such as malaria.

The immune response may be monitored by one of the following methods:

- An in vitro cellular response is determined by release of a relevant cytokine such as IFN- γ , from lymphocytes withdrawn from an animal or human being currently or
25 previously infected with malaria, or by detection of proliferation of these T cells. The induction being performed by the addition of the polypeptide or the immunogenic portion to a suspension comprising from 1×10^5 cells to 3×10^5 cells per well. The cells being isolated from either the blood, the spleen, the liver or the lung and the addition of the polypeptide or the immunogenic portion resulting in a concentration of not more than 20
30 μg per ml suspension and the stimulation being performed from two to five days. For monitoring cell proliferation the cells are pulsed with radioactive labeled Thymidine and after 16-22 hours of incubation detecting the proliferation by liquid scintillation counting. A positive response being a response more than background plus two standard deviations. The release of IFN- γ can be determined by the ELISA method, which is well known to a
35 person skilled in the art. A positive response being a response more than background plus

two standard deviations. Other cytokines than IFN- γ could be relevant when monitoring the immunological response to the polypeptide, such as IL-12, TNF- α , IL-4, IL-5, IL-10, IL-6, TGF- β . Another and more sensitive method for determining the presence of a cytokine (e.g. IFN- γ) is the ELISPOT method where the cells isolated from either the blood, the spleen, the liver or the lung are diluted to a concentration of preferable of 1 to 4×10^6 cells /ml and incubated for 18-22 hrs in the presence of of the polypeptide or the immunogenic portion resulting in a concentration of not more than 20 μ g per ml. The cell suspensions are hereafter diluted to 1 to 2×10^6 / ml and transferred to Maxisorp plates coated with anti-IFN- γ and incubated for preferably 4 to 16 hours. The IFN- γ producing cells are determined by the use of labelled secondary anti-IFN- γ antibody and a relevant substrate giving rise to spots, which can be enumerated using a dissection microscope. It is also a possibility to determine the presence of mRNA coding for the relevant cytokine by the use of the PCR technique. Usually one or more cytokines will be measured utilizing for example the PCR, ELISPOT or ELISA. It will be appreciated by a person skilled in the art that a significant increase or decrease in the amount of any of these cytokines induced by a specific polypeptide can be used in evaluation of the immunological activity of the polypeptide.

- An *in vitro* cellular response may also be determined by the use of T cell lines derived from an immune individual or a malaria infected person where the T cell lines have been driven with either live *P.falciparum*, extracts from the parasite or culture filtrate for 10 to 20 days with the addition of IL-2. The induction being performed by addition of not more than 20 μ g polypeptide per ml suspension to the T cell lines containing from 1×10^5 cells to 3×10^5 cells per well and incubation being performed from two to six days. The induction of IFN- γ or release of another relevant cytokine is detected by ELISA. The stimulation of T cells can also be monitored by detecting cell proliferation using radioactively labeled Thymidine as described above. For both assays a positive response being a response more than background plus two standard deviations.

- An *in vivo* cellular response which may be determined as a positive DTH response after intradermal injection or local application patch of at most 100 μ g of the polypeptide or the immunogenic portion to an individual who is clinically or subclinically infected with *P.falciparum*, a positive response having a diameter of at least 5 mm 72-96 hours after the injection or application.

35

- An *in vitro* humoral response is determined by a specific antibody response in an immune or infected individual. The presence of antibodies may be determined by an ELISA technique or a Western blot where the polypeptide or the immunogenic portion is absorbed to either a nitrocellulose membrane or a polystyrene surface. The serum is preferably diluted in PBS from 1:10 to 1:100 and added to the absorbed polypeptide and the incubation being performed from 1 to 12 hours. By the use of labeled secondary antibodies the presence of specific antibodies can be determined by measuring the OD e.g. by ELISA where a positive response is a response of more than background plus two standard deviations or alternatively a visual response in a Western blot.

10

- Another relevant parameter is measurement of the protection in animal models induced after vaccination with the polypeptide in an adjuvant or after DNA vaccination. Suitable animal models include primates, guinea pigs or mice, which are challenged with an infection. Readout for induced protection could be decrease of the parasite density compared to non-vaccinated animals, prolonged survival times compared to non-vaccinated animals and diminished weight loss compared to non-vaccinated animals.

15

Homologue protein

Homology is defined as an analogue or variant of the fusion protein of the present invention. The fusion protein is characterised by specific amino acids and is encoded by specific nucleic acid sequences. It will be understood that such sequences include analogues and variants produced by recombinant or synthetic methods wherein such polypeptide sequences have been modified by substitution, insertion, addition or deletion of one or more amino acid residues in the recombinant polypeptide and still be immunogenic in any of the biological assays described herein. Substitutions are preferably "conservative". Substitutions are preferably silent substitutions in the codon usage which will not lead to any change in the amino acid sequence, but may be introduced to enhance the expression of the protein. These are defined according to the following table. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other. The amino acids in the third column are indicated in one-letter code.

20

25

30

ALIPHATIC	Non-polar	GAP
		ILV
	Polar-uncharged	CSTM
		NQ
	Polar-charged	DE
		KR
AROMATIC		HFVY

Vaccine, protein

The invention pertains to a vaccine composition comprising a fusion protein according to the invention. In order to ensure optimum performance of such a vaccine composition it is preferred that it comprises an immunologically and pharmaceutically acceptable carrier, vehicle or adjuvant.

An effective vaccine, wherein a protein of the invention is recognized by the animal, will in an animal model be able to decrease parasite load in blood and target organs, prolong survival times and/or diminish weight loss after challenge with a malarial parasite, compared to non-vaccinated animals

Furthermore, the fusion protein of the invention may be coupled to a carbohydrate or a lipid moiety, e.g. a carrier, or a modified in other ways, e.g. being acetylated.

When produced in a microorganism the fusion protein of the invention will normally not be acetylated if no special measures are taken. The acetylation may be advantageous as acetylated polypeptides may be more stable in cell, blood or body and tissue fluids. Furthermore, the acetylation may confer the polypeptide with a structure and confirmation which mimics the structure and confirmation of the native *P. falciparum* antigen.

Suitable carriers are selected from the group consisting of a polymer to which the polypeptide(s) is/are bound by hydrophobic non-covalent interaction, such as a plastic, e.g. polystyrene, or a polymer to which the polypeptide(s) is/are covalently bound, such as a polysaccharide, or a polypeptide, e.g. bovine serum albumin, ovalbumin or keyhole

limpet haemocyanin. Suitable vehicles are selected from the group consisting of a diluent and a suspending agent. The adjuvant is preferably selected from the group consisting of dimethyldioctadecylammonium bromide (DDA), Quil A, poly I:C, aluminium hydroxide, Freund's incomplete adjuvant, IFN- γ , IL-2, IL-12, monophosphoryl lipid A (MPL),
5 Trehalose Dimycolate (TDM), Trehalose Dibehenate and muramyl dipeptide (MDP).

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231 and 4,599,230, all incorporated herein by reference.

10

Other methods of achieving adjuvant effect for the vaccine include use of agents such as aluminum hydroxide or phosphate (alum), synthetic polymers of sugars (Carbopol), aggregation of the protein in the vaccine by heat treatment, aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C.*
15 *parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed. Other possibilities involve the use of immune modulating substances such as cytokines or synthetic IFN- γ inducers such as poly I:C in
20 combination with the above-mentioned adjuvants.

Another interesting possibility for achieving adjuvant effect is to employ the technique described in Gosselin *et al.*, 1992 (19). In brief, a relevant antigen such as an antigen of the present invention can be conjugated to an antibody (or antigen binding antibody
25 fragment) against the Fc γ receptors on monocytes/macrophages.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the
30 individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 μ g to 1000 μ g, such as in the range from about 1 μ g to 300 μ g, and especially in the range from about 10 μ g to 50 μ g. Suitable regimens for initial administration and booster shots are also variable but
35 are typified by an initial administration followed by subsequent inoculations or other administrations.

5 The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated and, to a lesser degree, the size of the person to be vaccinated.

10 The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral
15 formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and advantageously contain 10-95% of active ingredient, preferably 25-70%.

20 In many instances, it will be necessary to have multiple administrations of the vaccine. Especially, vaccines can be administered to prevent an infection with malaria and/or to treat established malarial infection. When administered to prevent an infection, the vaccine is given prophylactically, before definitive clinical signs or symptoms of an
25 infection are present.

Due to genetic variation, different individuals may react with immune responses of varying strength to the same protein. Therefore, the vaccine according to the invention may comprise several different proteins in order to increase the immune response. The
30 vaccine may comprise two or more polypeptides or immunogenic portions, where all of the proteins are as defined above, or some but not all of the peptides may be derived from *P. falciparum* or other microorganisms. In the latter example, the polypeptides not necessarily fulfilling the criteria set forth above for polypeptides may either act due to
35 their own immunogenicity or merely act as adjuvants.

The vaccine may comprise 1-20, such as 2-20 or even 3-20 different proteines or fusion proteines, such as 3-10 different proteines or fusion proteines.

5 The invention also pertains to a method for immunising an animal, including a human being, against malaria caused by e.g. *P.falciparum*, comprising administering to the animal the fusion protein of the invention, or a vaccine composition of the invention as described above, or a living vaccine described below.

10 The invention also pertains to a method for producing an immunologic composition according to the invention, the method comprising preparing, synthesising or isolating a fusion protein according to the invention, and solubilizing or dispersing the fusion protein in a medium for a vaccine, and optionally adding other antigens and/or a carrier, vehicle and/or adjuvant substance.

15 Another aspect of the invention is producing the hybrid protein of the invention in a recombinant microorganism which, besides expressing the DNA sequence encoding the present hybrid protein, additionally expresses one or more antigens having a therapeutic or protective effect against another disease than malaria, e.g. tuberculosis. These other antigens can be expressed as separate antigens or as fused to the hybrid protein of the
20 present invention. Examples of other antigens effective against Tb are ESAT6, CFP7, CFP10, CFP29, ORF2c, TB13, MPT59, α -crystalline, Rv0285 and hybrids hereof, but the concept is not limited to TB or antigens against TB alone.

Vaccine DNA.

25 The nucleic acid fragments of the invention may be used for effecting *in vivo* expression of antigens, *i.e.* the nucleic acid fragments may be used in so-called DNA vaccines as reviewed in Ulmer et al 1993, which is included by reference.

30 Hence, the invention also relates to a vaccine comprising a nucleic acid fragment according to the invention, the vaccine effecting *in vivo* expression of antigen by an animal, including a human being, to whom the vaccine has been administered, the amount of expressed antigen being effective to confer substantially increased resistance to infections caused by *P.falciparum* in an animal, including a human being.

The efficacy of such a DNA vaccine can possibly be enhanced by administering the gene encoding the expression product together with a DNA fragment encoding a polypeptide which has the capability of modulating an immune response.

5 Live recombinant vaccines

One possibility for effectively activating a cellular immune response for a vaccine can be achieved by expressing the relevant antigen in a vaccine in a non-pathogenic microorganism or virus. Well-known examples of such microorganisms are *Mycobacterium bovis* BCG, *Salmonella* and *Pseudomona* and examples of viruses are
10 Vaccinia Virus and Adenovirus.

Therefore, another important aspect of the present invention is an additional quality of the living BCG vaccine presently available, wherein one or more copies of a DNA sequence encoding one or more fusion proteins as defined above has been incorporated into the
15 genome of the micro-organism in a manner allowing the micro-organism to express and secrete the protein. The incorporation of more than one copy of a nucleotide sequence of the invention is contemplated to enhance the immune response.

Another aspect of the invention is a non-pathogenic microorganism, such as e.g. *L. lactis* or BCG, expressing the DNA sequence encoding one or more fusion proteins as defined
20 above and additionally expressing one or more antigens having a therapeutic or protective effect against a disease different from malaria, such as e.g. tuberculosis caused by *Mycobacterium tuberculosis*. These other antigens can be expressed as separate antigens or as fused to the hybrid protein of the present invention. Examples of other antigens
25 effective against Tb (identified by their Sanger database accession number) are Rv3875 (ESAT6), Rv1886c (Ag85B), Rv0288 (CFP7), Rv3874 (CFP10), Rv0798c (CFP29), Rv2031c (α -crystalline) and Rv0285 or fragments or hybrids hereof most preferable the ESAT6-Ag85B hybrid, but the concept is not limited to TB or antigens against TB alone.

30 The effect of such a DNA-vaccine can possibly be enhanced by administering the gene encoding the expression product together with a DNA fragment encoding a polypeptide which has the capability of modulating an immune response. For instance, a gene encoding lymphokine precursors or lymphokines (e.g. INF- γ , IL-2, IL-12) could be administered together with the gene encoding the immunogenic fusion protein, either by
35 administering two separate DNA fragments or by administering both DNA fragments included in the same vector.

Another possibility is to integrate the DNA encoding the polypeptide according to the invention in an attenuated virus such as the vaccinia virus or Adenovirus (40). The recombinant vaccinia virus is able to replicate within the cytoplasm of the infected host cell and the protein of interest can therefore induce an immune response, which is envisioned to induce protection against malaria.

Therapeutic vaccine.

The invention also relates to the use of a fusion protein or nucleic acid of the invention for use as therapeutic vaccines as have been described in the literature exemplified by D. Lowry (Lowry et al 1999). Antigens with therapeutic properties may be identified based on their ability to diminish the severity of malarial infection in experimental animals or prevent reactivation of previous infection, when administered as a vaccine. The composition used for therapeutic vaccines can be prepared as described above for vaccines.

Legends to figures:

Figure 1. Schematic representation of pPSM1013 and pAMJ328 and the expression constructs used in *L. lactis*. The position of vector encoded restriction sites mentioned in the text, promoter P170, Shine-Dalgarno sequence (SD), and 310mut2 signal peptide are indicated. The signal peptidase is predicted to cleave between amino acid no. 32 and 33, thus leaving Ala-Glu residues in the N-terminal end of the mature recombinant proteins. The nucleotide numbering of *glurp* and *MSP3* was relative to A in the ATG codon of M59706 and L07944, respectively.

Figure 2. (A) Coomassie blue-stained 12.5% polyacrylamide gel of purified GLURP-MSP3 fusion protein (lane 1), GLURP₂₅₋₅₁₄ (lane 2), and MSP3₂₁₂₋₃₈₀ (lane 3) produced in *L. lactis* MG1363. (B) HPLC analysis on a C4 column of the GLURP-MSP3 hybrid protein and GLURP₂₅₋₅₁₄, respectively. The sizes (in kilodaltons) of the molecular mass markers are indicated. (C) Deduced amino acid sequences and peptide mapping of GLURP-MSP3 hybrid and GLURP₂₅₋₅₁₄. The first four amino acids (Ala-Glu-Arg-Ser) of the GLURP-MSP3 hybrid are derived from the cloning vector pSM1013. Samples for peptide mass mapping were cut out of a coomassie stained SDS-PAGE gel. Half a band (approx. 1 µg protein) was washed, dried, reduced and alkylated with

iodoacetamide before being digested overnight by modified trypsin (Promega, USA), essentially as described (44). The supernatant of the digest was applied to GELoader tips (Eppendorff, Germany) packed with Poros 20 R2 reversed phase material (PerSeptive, USA) and eluted with 0.8 µl of alpha-cyanohydroxycinnamic acid (20 µg/µl in 70% acetonitrile/30% water) directly onto the MALDI target (28). Analysis was carried out on a PerSeptive Voyager STR (PerSeptive, USA) operated in the reflector mode and the results were analyzed in GPMAW ver. 5.02 (Lighthouse data, Denmark). Sequences covered by peptides in the MALDI-TOF spectra are underlined and the percentage of total coverage of sequencing is indicated.

Figure 3. Patterns of IgG antibody responses to pairs of GLURP and MSP3 derived antigens in 71 plasma samples from adult Liberians clinically immune to malaria. The coefficient of correlation and P value are provided in each panel.

Figure 4. Antibody responses in mice. Groups of 10 mice were immunized with the hybrid (gr7), a mixture of GLURP and MSP3 in one syringe (gr8), or with GLURP and MSP3 in separate syringes at different sites (gr9). (A) Day 35 plasma samples were tested for antibody reactivity on ELISA plates coated with GLURP₂₅₋₅₁₄ or MSP3₂₁₂₋₃₈₀. Box plots show medians, 25th, and 75th percentiles and whiskers show the range of the data. (B) Cumulative responses of mouse sera with 8 peptides representing GLURP B-cell epitopes (51) and (C) isotype response of mice for which results are presented in panel A. Each vertical bar represents the mean absorbance (±SEM) in GLURP- and MSP3-specific ELISAs.

Figure 5. The hybrid contains only GLURP and MSP3 derived B-cell epitopes. A pool of plasma from mice immunized with the hybrid was pre-incubated with GLURP, MSP3, a mixture of GLURP and MSP3 or the hybrid at the indicated concentrations before being added to ELISA coated with the hybrid. Prior incubation with a mixture of GLURP and MSP3 or the hybrid completely inhibited Ig antibody binding to the hybrid.

Figure 6. Immunoblot analysis of *P. falciparum* NF54. A whole cell extract was separated on a 7.5% polyacrylamide gel and subjected to immunoblotting with plasma from mice immunized with GLURP₂₅₋₅₁₄ (lane 1), MSP3₂₁₂₋₃₈₀ (lane 2) and GLURP-MSP3 hybrid (lane 3). The sizes (in kilodaltons) of the molecular mass markers are indicated.

Examples

Example 1: Materials and methods

- 5 **Bacterial strains, plasmids and growth conditions.** *E. coli* DH10B (K-12, F⁺ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*dlacZ* Δ*m15* Δ*lacX74* *deoRrecA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ⁺ *rpsL nupG*) (Life Technologies) containing the indicated plasmids was grown in Luria broth (LB) supplemented with erythromycin (200 μg/ml). *L. lactis* MG1363 (17) containing the indicated plasmids was grown in either M17 broth
- 10 (Difco Ltd.) with 0.5 % (wt/vol) glucose or an enhanced synthetic amino acid (SA) medium named 3 x SA IV medium (24) supplemented with 1 μg/ml of erythromycin. Solidified LB or M17 media was supplemented with 200 or 1 μg/ml of erythromycin, respectively. The vector, pPSM1013 (Fig 1), is a high-copy number expression plasmid based on the pAMβ1 replicon (46) containing unique restriction sites allowing the
- 15 construction of in-frame fusions with an optimized secretion signal-peptide sequence, SP310mut2 (Ravn, P., Arnau, J., Madsen, S.M., Vrang, A., and Israelsen, H. unpublished). The mRNA for the peptide is translated from a plasmid-encoded translational start site and transcribed from the pH and growth phase inducible *L. lactis* promoter, P170 (7, 23, 33). There is essentially no transcription from the P170 promoter
- 20 at pH values of 7 or more. However, the transcription is induced in the transition to stationary phase at pH values below 6.5. Plasmid pAMJ328 is derived from pPSM1013 by deleting all *lacZ* regulatory sequences to avoid transcription from the *lac* promoter and by creating a new cloning region devoid of the signal peptide (32).
- 25 **Construction of plasmids expressing GLURP and MSP3 in *L. lactis*.** All plasmids were constructed in *E. coli* DH10B and transformed into *L. lactis* MG1363 by electroporation as described (22). All plasmid constructions were verified by DNA sequencing.
- 30 **pMST73.** The non-repeat region of FVO *glurp* was amplified with the primers 5'-CCC AGA TCT ACA AGT GAG AAT AGA AAT AAA C [nucleotides 79 to 100] (counting from A in the ATG start codon of M59706) and 5'-CCC AGA TCT TGC TTC ATG CTC GCT TTT TT CCG AT [nucleotides 1475 to 1500]; digested with *Bg*/II, and the resulting DNA fragment was cloned into *Bg*/II digested pPSM1013.
- 35 **pKBR5.** pMST73 plasmid was digested with *Bam*HI and *Sal*I, and the resulting DNA fragment containing the *glurp* insert was cloned into *Bam*HI-*Sal*I digested pAMJ328.

pKBR7. The non-repeat region of F32 *glurp* was amplified with the primers 5'-AAG TAG ATC TAC TAA TAC AAG TGA GAA TAG AAA TAA AC [nucleotides 73 to 100], and 5'-GTT CAG ATC TTT ATT CAT GAT GGC CTT CTA GC [nucleotides 1519 to 1542]; the resulting DNA fragment digested with *Bg*/II and cloned into *Bg*/II digested pPSM1013.

pKBR8. Plasmid pKBR7 was digested with *Bam*HI and *Sa*II, and the *glurp* insert was cloned into *Bam*HI-*Sa*II digested pAMJ328.

pKBR9. The C-terminal region of F32 *MSP3* was amplified with the primers 5'-CCC AGA TCT AAA GCA AAA GAA GCT TCT AGT TAT [nucleotides 628 to 651] and 5'-ATT AGA TCT CAT TTA ATG ATT TTT AAA ATA TTT GGA TA, [nucleotides 1118 to 1140] (counting from A in the ATG start codon of L07944); the resulting DNA fragment was digested with *Bg*/II and cloned into *Bg*/II digested pPSM1013. This *MSP3* region is identical to that of the FC27 allele (Accession number L07944) except for the following residues at variable positions in *MSP3*: 735 (T → C) and 948 (A → G).

pKBR10. Plasmid pKBR9 was digested with *Bam*HI and *Sa*II, and the *MSP3* insert was cloned into *Bam*HI-*Sa*II digested pAMJ328.

pKBR11. The *Bg*/II-fragment of pKBR9 was cloned into pKBR5 digested partially with *Bg*/II yielding an in frame fusion between *glurp*₇₉₋₁₅₀₀ and *MSP3*₆₂₈₋₁₁₄₀. This hybrid molecule corresponds to the F32 allele except for the following residues at variable positions in GLURP: Leu-50, Asn-53, Glu-65, Asp-129, Glu-224, Pro-500.

Fermentation. Fermentation of *L. lactis* MG1363, containing plasmid pKBR8 (GLURP), pKBR10 (*MSP3*) or pKBR11 (GLURP-*MSP3* hybrid), was carried out in 1 L of 3xSA IV-media supplemented with erythromycin (1 µg/ml), yeast-extract (0.5%) and glucose (1.5%) in 2 L fermentors at 30°C. The starting pH of the culture medium was adjusted to 7.4. Since *L. lactis* MG1363 produces lactic acid during the growth, pH is declining as cell density increases. After approximately 3 hours of growth, pH was reduced to 6 and this level was maintained by a pH-controlled intake of 2 M KOH for another 8 hours until the cell density was approximately OD₆₀₀ = 8. A 50% glucose solution was added in parallel with the base since this tends to increase the bacterial yield. Bacterial cells were removed from the culture-supernatant (containing exported protein) by ultrafiltration with a Pellicon 2 Durapore filter (PVDF, 0.22 µm, 0.1 m²) (Millipore). Culture-supernatants were either used immediately or stored at -20 °C.

Purification of recombinant proteins. A purification strategy was developed for the recombinant GLURP, *MSP3* and hybrid molecules. Cell-free culture-supernatants were

concentrated on a Millipore Labscale™ TFF System installed with a Pellicon XL Biomax 8 filter (Polypropylene-membrane, 50000 Da, 50 cm²) and concentrates were buffer exchanged to 20 mM Bis-Tris (pH 6.4) on a Sephadex G-25 column (C26/40, 170 ml). Recombinant proteins were first purified on a 5 ml HiTrap Q Sepharose High Performance (Pharmacia Biotech) column by applying a gradient of 0 to 1 M NaCl in column buffer at a flow-rate of 1 ml/min. Fractions (2 ml) containing the desired recombinant protein were pooled and dialyzed against 20 mM Bis-Tris (pH 6.4) and applied to a 5 ml HiTrap SP Sepharose High Performance (Pharmacia Biotech) column. The recombinant protein was eluted by a gradient of 0 to 1 M NaCl in column buffer. GLURP and MSP3 were eluted in single peaks whereas the hybrid was eluted in two peaks. Fractions (2 ml) containing the desired peaks were pooled and adjusted to 1 M (NH₄)₂SO₄ and further purified on a 5 ml Phenyl Sepharose High Performance (Pharmacia Biotech) by applying a gradient of 1 to 0 M (NH₄)₂SO₄ in 20 mM Bis-Tris (pH 6.4) at a flow-rate of 1 ml/min. Analysis of all fractions was performed by SDS-PAGE. Protein concentrations were measured by the BCA™ protein assay (Pierce, Rockford, Illinois, USA).

Immunization and purification of mouse IgG. Thirty BALBc/CF1 BALBc/CF1 mice (27) female mice (7 to 10 weeks of age) were randomly assigned to three groups. Two groups were immunized with 20 µg of GLURP₂₇₋₅₀₀-MSP3₂₁₂₋₃₈₀ hybrid (gr7), or with a mixture of 15 µg GLURP₂₅₋₅₁₂ and 5 µg MSP3₂₁₂₋₃₈₀ (gr8) by subcutaneous injections at the base of the tail, respectively; and the third group (gr9) received 15 µg GLURP₂₅₋₅₁₂ injected at the base of the tail and 5 µg MSP3₂₁₂₋₃₈₀ injected in the shoulder. All immunogens were emulsified in Montanide (® - ?) and each mouse received three injections at 2-week intervals and was bled on days 0, 14, 28 and 35. Total IgG was purified by (NH₄)₂SO₄ precipitation and subsequent purification on DEAE-columns from pooled serum samples taken on day 35 from animals in the groups gr7, 8, and 9 and from pooled day 0 samples.

ELISA and serum samples. Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described in detail (54). The coating concentrations of GLURP₂₅₋₅₁₂, MSP3₂₁₂₋₃₈₀, and GLURP₂₇₋₅₀₀-MSP3₂₁₂₋₃₈₀ were 0.5, 1.0 and 0.5 µg/ml respectively. Serial dilutions of plasma from Liberian adults clinically immune to malaria, Danish donors never exposed to malaria (51), and mice were tested on ELISA plates coated with either antigen and the absorbance values were plotted against the plasma dilutions. In order to compare anti-hybrid antibody responses with the respective

anti-GLURP and anti-MSP3 antibody responses in different plasma samples the antibody titer was defined as the plasma dilution, which gives an absorbance value of $A_{492} = 1000$ in the parallel portion of the curves.

5 **Competition ELISA assays.** Recombinant GLURP₂₅₋₅₁₈ and MSP3₂₁₂₋₃₈₀ and a mixture of these two antigens were added at various concentrations (3.2×10^{-5} µg/ml to 100 µg/ml) to a pool of plasma from mice immunized with the GLURP-MSP3 hybrid diluted in 1.25% (w/v) milk powder in PBS. The plasma dilution used was adjusted to give an absorbance (A_{492}) of approximately 2500. The antigen-antibody mixtures were
10 incubated overnight at 4 °C and subsequently the reactivity to GLURP-MSP3 hybrid coated ELISA plates was determined.

Indirect Immunofluorescent Antibody (IFA) test. IFA was performed as reported earlier (5). Briefly, a thin film of RBCs containing predominantly schizonts stages of *P. falciparum* NF54 were incubated with serial dilutions of purified mouse IgG in
15 phosphate buffered saline (PBS pH 7.4) for 30 min at 37°C in a humid chamber. After washing with PBS, mouse antibodies were revealed with Alexa Fluor conjugated goat anti-mouse IgG (Molecular probe, USA) diluted 1:300 in PBS. After washing the slide was examined under UV light. The endpoint titre was the highest dilution of the
20 antibodies, which produce visible specific immunofluorescence.

RP-HPLC analysis of GLURP and GLURP-MSP3. Samples were analyzed on a HPLC system (Pharmacia, Sweden), using a Protein C4 column (VYDAC®, 214TP54, USA). Analysis was done in a Acetonitrile : H₂O : TFA buffer system. Purified samples
25 were diluted 1:2 in A-buffer (H₂O + 0,1 % (w/v) TFA) and applied on the column, elution was done using a linear gradient 0-80% B-buffer (80 % Acetonitrile + 0.1 % (w/v) TFA) over 20 column volumes. Elution was monitored by UV-Abs. 214 nm. Peaks were collected and vacuum dried on a HetoVac (Heto, Denmark) and kept on 4 °C until further experiments.

30 **Maldi-Tof MS and ES-MS.** Samples for peptide mass mapping for were cut out of a coomassie stained SDS-PAGE gel. Half a band (approx. 1 µg protein) was washed, dried, reduced and alkylated with iodoacetamide before being digested overnight by modified trypsin (Promega, USA), essentially as described (44). The supernatant of the digest was applied to GELoader tips (Eppendorff, Germany) packed with Poros 20 R2
35 reversed phase material (PerSeptive, USA) and eluted with 0.8 µl of alpha-cyanohydroxycinnamic acid (20 µg/µl in 70% acetonitrile/30% water) directly onto

the MALDI target (28). Analysis was carried out on a PerSeptive Voyager STR (PerSeptive, USA) operated in the reflector mode and the results were analyzed in GPMAW ver. 5.02 (Lighthouse data, Denmark).

Electrospray mass spectrometry of the intact protein was carried out on a fraction from RP-HPLC (approx. 20 µg protein). The sample was dried down and re-dissolved in 5% formic acid to a concentration of 20 pmol/µl before being analyzed on a Micromass QTOF (Micromass, UK) using a nanospray source.

Example 2: Expression of *glurp* and *MSP3* in *L. lactis*.

PCR fragments encoding the *glurp*₇₉₋₁₅₀₀ and *MSP3*₆₂₈₋₁₁₄₀ regions were cloned side by side thereby creating an in-frame fusion between a vector-encoded signal-peptide and a GLURP₂₇₋₅₀₀-MSP3₂₁₂₋₃₈₀ fusion protein (pKBR11, Fig. 1). This hybrid contains two additional amino acid residues created by joining these *glurp* and *MSP3* fragments. For comparison, the individual *glurp*₇₃₋₁₅₄₂ and *MSP3*₆₂₈₋₁₁₄₀ fragments were also cloned (pKBR8 and pKBR10, Fig. 1). Plasmids were transformed into *L. lactis* MG1363 and the resulting strains were grown in fermentors as described in Materials and Methods. The pH of the growth medium was maintained at 6 to achieve optimal transcription from the P170 promoter (33). All three recombinant proteins were secreted into the culture supernatants from where they were purified by sequential ion exchange on HiTrap Q and SP Sepharose columns followed by hydrophobic interaction chromatography on Phenyl Sepharose. Subsequent SDS-PAGE showed that the plasmids pKBR11 (lane 1), pKBR8 (lane 2), and pKBR10 (lane 3) produced major products of 136, 100, and 36 kDa respectively (Fig. 2A). Additional lower molecular-mass bands were observed in the purified GLURP and MSP3 preparations. When analyzed by immunoblotting the smaller products in lanes 2 and 3 were specifically recognized, as were the full-length products, by antibodies to GLURP and MSP3 respectively, suggesting that they may result from incomplete translation of the mRNA and/or from protease cleavage of the primary protein products. A MALDI MS tryptic peptide map of the SDS-PAGE purified bands in lane 2 confirmed that this smaller molecular-mass protein is derived from GLURP₂₅₋₅₁₄ (data not shown). The purity of the GLURP₂₇₋₅₀₀-MSP3₂₁₂₋₃₈₀ and GLURP₂₅₋₅₁₄ preparations was assessed by HPLC as described in Materials and Methods. GLURP₂₇₋₅₀₀-MSP3₂₁₂₋₃₈₀ and GLURP₂₅₋₅₁₄ gave single major peaks (Fig. 2B). The molecular masses of GLURP₂₇₋₅₀₀-MSP3₂₁₂₋₃₈₀ and GLURP₂₅₋₅₁₄ were 74950 and 56518 Da (± 20 Da), respectively, as determined by ES MS. Assuming that the two recombinant proteins each contain the vector encoded amino acid residues, A-E-R-S, attached to their N-terminal ends (Fig. 1), these

molecular weights corresponds well to the predicted values of 74939 and 56518, respectively. Thus, both GLURP₂₇₋₅₀₀-MSP3₂₁₂₋₃₈₀ and GLURP₂₅₋₅₁₄ recombinant proteins were intact and contained the predicted amino acid residues.

5 Example 3: Antigenicity of GLURP and MSP3 produced in *L. lactis*.

The antigenicity of the recombinant proteins was evaluated against plasma from 71 adults Liberians clinically immune to malaria (Fig. 3). Serial dilutions of all plasma samples were tested on separate plates coated with each recombinant protein and the antigen-specific titer was determined as the dilution giving an absorbance of 1000. As
10 expected, different plasma contained different amounts of GLURP and MSP3-specific IgG antibodies (Fig. 3A). In general, hybrid-specific antibody titers exceeded those recorded with the individual GLURP₂₅₋₅₁₄ and MSP3 antigens (Fig. 3B and C) suggesting that the hybrid molecule provides an adequate presentation of GLURP and MSP3 antigenic determinants, respectively.

15

Example 4: Immunogenicity of recombinant GLURP and MSP3 products.

To determine whether the GLURP-MSP3 hybrid molecule is a superior immunogen compared to a mixture of the individual GLURP₂₅₋₅₁₄ and MSP3₂₁₂₋₃₈₀ molecules, groups of BALBc/CF1 mice were each immunized subcutaneously with the hybrid molecule in
20 Montanide or with GLURP₂₅₋₅₁₄ and MSP3₂₁₂₋₃₈₀ combined in either one syringe or injected separately at two different sites. Following the third injection, day-35 sera were tested for IgG antibody reactivity against GLURP and MSP3, respectively. While the mean GLURP-ELISA titer is only marginally higher in the hybrid group than in the other two groups, mean MSP3-ELISA titer is 4.3-fold higher (Kruskal Wallis test,
25 $P < 0.004$) in the group receiving the hybrid compared to the group receiving MSP3₂₁₂₋₃₈₀ and GLURP₂₅₋₅₁₄ at two different sites (compare gr7 and gr9 in Fig. 4A). At the individual level, mice immunized with the hybrid reacted strongly with both GLURP and MSP3 domains whereas mice immunized with a combination of two molecules tended to mount a predominant antibody response against either GLURP or MSP3. The
30 anti-hybrid IgG antibodies are mainly directed against the P3, P4, P11, and S3 peptides containing known epitopes for human antibodies (51); however peptides P5 and P9 which do not contain such epitopes were also recognized (Fig. 4B). Whereas the GLURP and MSP3-specific IgG subclass profiles are similar for all vaccine formulations (Fig. 4C), GLURP-specific IgG antibodies tends to use the Kappa light
35 chain and MSP3-specific IgG antibodies tends to use the Lambda light chain. This

difference in light chain was found for all GLURP or MSP3-specific antibodies whether raised against the hybrid or the mixtures of the individual molecules.

The specificity of mouse antibodies to the hybrid was also analyzed by competition-ELISA (Fig. 5). It appears that antibodies to the hybrid are purely GLURP and MSP3-specific, since a mixture of soluble GLURP₂₅₋₅₁₄ and MSP3₂₁₂₋₃₈₀ could completely inhibit the binding of anti-hybrid antibodies to immobilized GLURP₂₇₋₅₀₀-MSP3₂₁₂₋₃₈₀. Thus, the construction of a GLURP-MSP3 hybrid molecule has not created new B-cell epitopes in the overlapping area.

10 Example 5: Reactivity of mouse anti-GLURP and anti-MSP3 sera with native GLURP and MSP3.

The immunogenicity of the recombinant GLURP and MSP3 was also investigated by immunoblotting of parasite-derived proteins with sera from mice immunized with each of the three recombinant proteins, hybrid, GLURP₂₅₋₅₁₄ and MSP3₂₁₂₋₃₈₀, respectively. As demonstrated in Fig. 6, plasma from mice immunized with GLURP₂₅₋₅₁₄, MSP3₂₁₂₋₃₈₀, and the hybrid recognized polypeptides of approximately 220,000 Da (lane 1), 48,000 Da (lane 2), and both (lane 3), respectively.

20 Example 6: Antigen competition between GLURP and MSP3 produced as long synthetic peptides.

Immunogens

The MSP3 and GLURP regions used were produced as long synthetic peptides:

MSP3 (LR55) : 181-RKTKEYAEKA KNAYEKAKNA YQKANQAVLK AKEASSYDYI
25 LGWEFGGGVP EHKKEENMLS HLYVSSKDKE NISKENDDLV DEKEEEAEET EEEEELE-
276 .

and

GLURP (LR67) : 85 NVPSGL DIDDIPKESI FIQEDQEGQT HSELNPETSE HSKDLNNGS
KNESDIISE NNKSNKVQNH FESLSDELL ENSSQDNLDK DTISTEPFPN
30 QKHKDLQQDL NDEPLEPFPT QHKDYKEKN LIN-213.

Immunizations

Twenty BALBc female mice (7 to 10 weeks of age) were randomly assigned to four groups and immunized by subcutaneous injections with different combinations of MSP3 and GLURP:
35

1. group 110 was immunized with 5 µg of LR55,

2. group 111 was immunized with 5 µg of LR67,
3. group 112 was immunized with a mixture of 5 µg LR55 + 5 µg LR67 by subcutaneous injections at the base of the tail,
4. group 113 received 5 µg LR55 injected at the base of the tail and 5 µg LR67 injected in the shoulder.

All immunogens were emulsified in Montanide ISA720 and each mouse received three injections at 2-week intervals and was bled on days 0, 14, 28 and 35.

ELISA

Serial dilutions of day 35 plasma samples were tested on ELISA plates coated with either LR55 or LR67 at 0.5 µg/ml respectively, and the absorbance values were plotted against the plasma dilutions. The antibody titer was defined as the plasma dilution, which gives an absorbance value of A₄₉₂ = 1.00 in the parallel portion of the curves.

Results

To determine whether it is feasible to obtain a balanced immune response against a mixture of MSP3 and GLURP produced as long synthetic peptides, four groups of BALBc mice were each immunized subcutaneously with 1) LR55 (gr110), 2) LR67 (gr111), 3) LR55 and LR67 combined in one syringe (gr 112) or 4) LR55 and LR67 injected separately at two different sites (gr113). Sera collected 35 days after the first injection, were tested for IgG antibody reactivity against GLURP and MSP3, respectively. Mice immunized with LR55 or LR67 alone reacted strongly with either LR55 or LR67, respectively (Figure 7(a) and 7(b)). Likewise, mice immunized with the two molecules injected at different sites reacted strongly with both GLURP and MSP3 domains (Figure 7(d)) whereas mice immunized with a combination of the two molecules administered in one syringe reacted exclusively against LR55 (Figure 7(c)).

This result strongly supports the notion that a mixture of individual GLURP and MSP3 products cannot be administered in a single vaccine formulation without antigen competition between GLURP and MSP3.

References

1. Aribot, G., C. Rogier, J. L. Sarthou, J. F. Trape, A. T. Balde, P. Druilhe, and C. Roussillon. 1996. Pattern of immunoglobulin isotype response to *Plasmodium falciparum* blood-stage antigens in individuals living in a holoendemic area of Senegal (Dielmo, west Africa). *Am.J.Trop.Med.Hyg.* 54:449-457.
2. Aucan, C., Y. Traore, F. Tall, B. Nacro, T. Traore-Leroux, F. Fumoux, and P. Rihet. 2000. High immunoglobulin G2 (IgG2) and low IgG4 levels are associated with human resistance to *Plasmodium falciparum* malaria. *Infect.Immun.* 68:1252-1258.
3. Badell, E., C. Oeuvray, A. Moreno, Soe Soe, N. v. Rooijen, A. Bouzidi, and P. Druilhe. 2000. Human malaria in immunocompromised mice: an in vivo model to study defence mechanisms against *Plasmodium falciparum*. *J.Exp.Med.* 192:1653-1659.
4. Boudin, C., B. Chumpitazi, M. Dziegiel, F. Peyron, S. Picot, B. Hogh, and P. Ambroise-Thomas. 1993. Possible role of specific immunoglobulin M antibodies to *Plasmodium falciparum* antigens in immunoprotection of humans living in a hyperendemic area, Burkina Faso. *J.Clin.Microbiol.* 31:636-641.
5. Bouharoun-Tayoun, H., P. Attanath, A. Sabchareon, T. Chongsuphajaisiddhi, and P. Druilhe. 1990. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. *J.Exp.Med.* 172:1633-1641.
6. Bouharoun-Tayoun, H., C. Oeuvray, F. Lunel, and P. Druilhe. 1995. Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. *J.Exp.Med.* 182:409-418.
7. Bredmose, L., S. M. Madsen, A. Vrang, P. Ravn, M. G. Johnsen, J. Glenting, J. Arnau, and H. Israelsen. 2001. Development of a heterologous geneexpression system for use in *Lactococcus lactis*, p. 269-275. In O.-W.Merten (ed.), *Recombinant Protein Production with Prokaryotic and Eukaryotic Cells*. Kluwer Academic Publishers.
8. Carvalho, L. J. M. 2000. Instituto Oswaldo Cruz/Fiocruz. Evaluation of the immunogenicity and protective efficacy of *Plasmodium falciparum* MSP3 and GLURP in the neotropical primates *Saimiri sciureus* and *Aotus infulatus*.
9. Clark, J. T., S. Donachie, R. Anand, C. F. Wilson, H. G. Heidrich, and J. S. McBride. 1989. 46-53 kilodalton glycoprotein from the surface of *Plasmodium falciparum* merozoites. *Mol.Biochem.Parasitol.* 32:15-24.
10. Cohen, S., A. McGregor, and S. Carrington. 1961. Gamma globulin and aquired immunity to human malaria. *Nature* 192:733-737.
11. Dodoo, D., M. Theisen, J. A. Kurtzhals, B. D. Akanmori, K. A. Koram, S. Jepsen, F. K. Nkrumah, T. G. Theander, and L. Hviid. 2000. Naturally acquired antibodies to the glutamate-rich protein are associated with protection against *Plasmodium falciparum* malaria. *J.Infect.Dis.* 181:1202-1205.

12. **Druilhe, P. and J. L. Perignon . 1994.** Mechanisms of defense against *P. falciparum* asexual blood stages in humans. *Immunol.Lett.* **41**:115-120.
13. **Druilhe, P., A. Sabchareon, H. Bouharoun-Tayoun, C. Oeuvray, and J. L. Perignon. 1997.** In vivo veritas: lessons from immunoglobulin-transfer experiments in malaria patients. *Ann.Trop.Med.Parasitol.* **91, Supp.**:37-53.
14. **Druilhe, P., A. Sabchareon, H. Bouharoun-Tayoun, C. Oeuvray, and J. L. Perignon. 1997.** In vivo veritas: lessons from immunoglobulin-transfer experiments in malaria patients. *Ann.Trop.Med.Parasitol.* **91, Supp.**:37-53.
15. **Dziegiel, M., P. Rowe, S. Bennett, S. J. Allen, O. Olerup, A. Gottschau, M. Borre, and E. M. Riley. 1993.** Immunoglobulin M and G antibody responses to *Plasmodium falciparum* glutamate-rich protein: correlation with clinical immunity in Gambian children. *Infect.Immun.* **61**:103-108.
16. **Epping, R. J., S. D. Goldstone, L. T. Ingram, J. A. Upcroft, R. Ramasamy, J. A. Cooper, G. R. Bushell, and H. M. Geysen. 1988.** An epitope recognised by inhibitory monoclonal antibodies that react with a 51 kilodalton merozoite surface antigen in *Plasmodium falciparum*. *Mol.Biochem.Parasitol.* **28**:1-10.
17. **Gasson, M. J. 1983.** Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J.Bacteriol.* **154**:1-9.
18. **Genton, B., F. Al Yaman, R. Anders, A. Saul, G. Brown, D. Pye, D. O. Irving, W. R. Briggs, A. Mai, M. Ginny, T. Adiguma, L. Rare, A. Giddy, R. Reber-Liske, D. Stuerchler, and M. P. Alpers. 2000.** Safety and immunogenicity of a three-component blood-stage malaria vaccine in adults living in an endemic area of Papua New Guinea. *Vaccine* **18**:2504-2511.
19. **Gosselin, E. J., K. Wardwell, D. R. Gosselin, N. Alter, J. L. Fisher, and P. M. Guyre. 1992.** Enhanced antigen presentation using human Fc gamma receptor (monocyte/macrophage)-specific immunogens. *J.Immunol.* **149**:3477-3481.
20. **Hisaeda, H., A. Saul, J. J. Reece, M. C. Kennedy, C. A. Long, L. H. Miller, and A. Stowers. 2002.** Merozoite surface protein 3 and protection against malaria in *Aotus nancymai* monkeys. *Journal of Infectious diseases* **185**:657-664.
21. **Hogh, B., E. Petersen, M. Dziegiel, K. David, A. Hanson, M. Borre, A. Holm, J. Vuust, and S. Jepsen. 1992.** Antibodies to a recombinant glutamate-rich *Plasmodium falciparum* protein: evidence for protection of individuals living in a holoendemic area of Liberia. *Am.J.Trop.Med.Hyg.* **46**:307-313.
22. **Holo, H. and I. F. Nes. 1995.** Transformation of *Lactococcus* by electroporation. *Methods Mol.Biol.* **47**:195-9.:195-199.
23. **Israelsen, H., S. M. Madsen, A. Vrang, E. B. Hansen, and E. Johansen. 1995.** Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn917-lacZ integrants with the new promoter probe vector, pAK80. *Appl.Environ.Microbiol.* **61**:2540-2547.
24. **Jensen, P. R. and K. Hammer. 1993.** Minimal requirements for exponential growth in *Lactococcus lactis*. *Appl.Environ.Microbiol.* **59**:4363-4366.

25. Keitel, W. A., K. E. Kester, R. L. Atmar, A. C. White, N. H. Bond, C. A. Holland, U. Krzych, D. R. Palmer, A. Egan, C. Diggs, W. R. Ballou, B. F. Hall, and D. Kaslow. 1999. Phase I trial of two recombinant vaccines containing the 19kd carboxy terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 (msp-1(19)) and T helper epitopes of tetanus toxoid. *Vaccine* 18:531-539.
26. Khusmith, S. and P. Druilhe. 1983. Antibody-dependent ingestion of *P. falciparum* merozoites by human blood monocytes. *Parasite Immunol.* 5:357-368.
27. Klausen, J., M. Magnusson, A. B. Andersen, and C. Koch. 1994. Characterization of purified protein derivative of tuberculin by use of monoclonal antibodies: isolation of a delayed-type hypersensitivity reactive component from *M. tuberculosis* culture filtrate. *Scand.J.Immunol.* 40:345-349.
28. Kussmann, M., U. Lassing, C. A. Sturmer, M. Przybylski, and P. Roepstorff. 1997. Matrix-assisted laser desorption/ionization mass spectrometric peptide mapping of the neural cell adhesion protein neurolin purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis or acidic precipitation. *J.Mass Spectrom.* 32:483-493.
29. Lawrence, G., Q. Q. Cheng, C. Reed, D. Taylor, A. Stowers, N. Cloonan, C. Rzepczyk, A. Smillie, K. Anderson, D. Pombo, A. Allworth, D. Eisen, R. Anders, and A. Saul. 2000. Effect of vaccination with 3 recombinant asexual-stage malaria antigens on initial growth rates of *Plasmodium falciparum* in non-immune volunteers. *Vaccine* 18:1925-1931.
30. Locher, C. P. and L. Q. Tam. 1993. Reduction of disulfide bonds in *Plasmodium falciparum* gp195 abolishes the production of growth-inhibitory antibodies. *Vaccine* 11:1119-1123.
31. Lunel, F. and P. Druilhe. 1989. Effector cells involved in nonspecific and antibody-dependent mechanisms directed against *Plasmodium falciparum* blood stages in vitro. *Infect.Immun.* 57:2043-2049.
32. Madsen, S. M. 2000. The Technical University of Denmark. Characterization of regulated promoters from *Lactococcus*.
33. Madsen, S. M., J. Arnau, A. Vrang, M. Givskov, and H. Israelsen. 1999. Molecular characterization of the pH-inducible and growth phase-dependent promoter P170 of *Lactococcus lactis*. *Mol.Microbiol.* 32:75-87.
34. McColl, D. J. and R. F. Anders . 1997. Conservation of structural motifs and antigenic diversity in the *Plasmodium falciparum* merozoite surface protein-3 (MSP-3). *Mol.Biochem.Parasitol.* 90:21-31.
35. McColl, D. J., A. Silva, M. Foley, J. F. Kun, J. M. Favaloro, J. K. Thompson, V. M. Marshall, R. L. Coppel, D. J. Kemp, and R. F. Anders. 1994. Molecular variation in a novel polymorphic antigen associated with *Plasmodium falciparum* merozoites. *Mol.Biochem.Parasitol.* 68:53-67.
40. Oeuvray, C., H. Bouharoun-Tayoun, H. Gras-Masse, E. Bottius, T. Kaidoh, M. Aikawa, M. C. Filgueira, A. Tartar, and P. Druilhe. 1994. Merozoite surface protein-3: a malaria protein inducing antibodies that promote *Plasmodium falciparum* killing by cooperation with blood monocytes. *Blood* 84:1594-1602.

37. Oeuvray, C., Roussilhon, C., Perignon, J. L., Sarthou, J. L., Cisse, B., Tall, A., Diagne, N., and Druilhe, P. Natural immunity against *falciparum* malaria is strongly associated with IgG3 antibodies against the merozoite surface protein-3, in an age-independent manner. 2000. Cartagena, Colombia, XVth International Congress for Tropical Medicine and Malaria.
5 Ref Type: Conference Proceeding
38. Oeuvray, C., M. Theisen, C. Rogier, J. F. Trape, S. Jepsen, and P. Druilhe. 2000. Cytophilic immunoglobulin responses to *Plasmodium falciparum* glutamate-rich protein are correlated with protection against clinical malaria in Dielmo, Senegal. Infect.Immun. 68:2617-2620.
10
39. Okenu, D. M. N., A. W. Thomas, and D. J. Conway. 2000. Allelic lineages of the merozoite surface protein 3 gene in *Plasmodium reichenowi* and *Plasmodium falciparum*. Mol.Biochem.Parasitol. 109:185-188.
40. Rolph, M. S. and I. A. Ramshaw. 1997. Recombinant viruses as vaccines and immunological tools. curr.Opin.Immunol. 9:517-524.
15
41. Sabchareon, A., T. Burnouf, D. Ouattara, P. Attanath, H. Bouharoun-Tayoun, P. Chantavanich, C. Foucault, T. Chongsuphajaisiddhi, and P. Druilhe. 1991. Parasitologic and clinical human response to immunoglobulin administration in *falciparum* malaria. Am.J.Trop.Med.Hyg. 45:297-308.
42. Sabchareon, A., T. Burnouf, D. Ouattara, P. Attanath, H. Bouharoun-Tayoun, P. Chantavanich, C. Foucault, T. Chongsuphajaisiddhi, and P. Druilhe. 1991. Parasitologic and clinical human response to immunoglobulin administration in *falciparum* malaria. Am.J.Trop.Med.Hyg. 45:297-308.
20
43. Saul, A., G. Lawrence, A. Smillie, C. M. Rzepczyk, C. Reed, D. Taylor, K. Anderson, A. Stowers, R. Kemp, A. Allworth, R. F. Anders, G. V. Brown, D. Pye, P. Schoofs, D. O. Irving, S. L. Dyer, G. C. Woodrow, W. R. Briggs, R. Reber, and D. Sturchler. 1999. Human phase I vaccine trials of 3 recombinant asexual stage malaria antigens with Montanide ISA720 adjuvant. Vaccine 17:3145-3159.
25
44. Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal.Chem. 68:850-858.
30
45. Shi, Y. P., B. L. Nahlen, S. Kariuki, K. B. Urdahl, P. D. McElroy, J. M. Roberts, and A. A. Lal. 2001. Fc Receptor IIa (CD32) polymorphism is associated with protection of infants against high-density *Plasmodium falciparum* infection. VII. Asembo Bay Cohort Project. J.Infect.Dis. 184:107-111.
35
46. Simon, D. and A. Chopin. 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie 70:559-566.
47. Soe Soe. 2000. Application of the antibody dependent cellular inhibition (ADCI) assay to the identification of protective antigens and the study of the establishment of protective immunity in Myanmar.
40
48. Stricker, K., J. Vuust, S. Jepsen, C. Oeuvray, and M. Theisen. 2000. Conservation and heterogeneity of the Glutamate-rich protein (GLURP) among field

- isolates and laboratory lines of *Plasmodium falciparum*. Mol.Biochem.Parasitol. 111:123-130.
49. Theisen, M., G. Cox, B. Høgh, S. Jepsen, and J. Vuust. 1994. Immunogenicity of the *Plasmodium falciparum* glutamate-rich protein expressed by vaccinia virus. Infect.Immun. 62:3270-3275.
 50. Theisen, M., D. Dodo, A. T. Balde, Soe Soe, Corradin G, K. A. Koram, J. Kurtzhals, T. G. Theander, B. D. Akanmori, G. Ndiaye, and P. Druilhe. 2001. Selection of long GLURP synthetic peptides for vaccine development: antigenicity, relationship with clinical protection and immunogenicity. Infect.Immun. 69:5223-5229.
 51. Theisen, M., Soe Soe, S. Jessing, L. Okkels, S. Danielsen, C. Oeuvray, P. Druilhe, and S. Jepsen. 2000. Identification of a major linear B cell epitope of the *Plasmodium falciparum* Glutamate-rich protein (GLURP), targeted by human antibodies mediating parasite killing. Vaccine 19:204-212.
 52. Theisen, M., Soe Soe, C. Oeuvray, A. W. Thomas, J. Vuust, S. Danielsen, S. Jepsen, and P. Druilhe. 1998. The glutamate-rich protein (GLURP) of *Plasmodium falciparum* is a target for antibody-dependent monocyte-mediated inhibition of parasite growth in vitro. Infect.Immun. 66:11-17.
 53. Theisen, M., A. W. Thomas, and S. Jepsen. 2001. Nucleotide sequence and analysis of the gene encoding the glutamate-rich protein (GLURP) from *Plasmodium reichenowi*. Mol.Biochem.Parasitol. 115:269-273.
 54. Theisen, M., J. Vuust, A. Gottschau, S. Jepsen, and B. Høgh. 1995. Antigenicity and immunogenicity of recombinant glutamate-rich protein of *Plasmodium falciparum* expressed in *Escherichia coli*. Clin.Diagn.Lab.Immunol. 2:30-34.
 55. Thomas, A. W., J. A. Deans, G. H. Mitchell, T. Alderson, and S. Cohen. 1984. The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit *Plasmodium knowlesi* invasion of erythrocytes. Mol.Biochem.Parasitol. 13:187-199.
 56. Warmerdam, P. A., J. G. van de Winkel, A. Vlug, N. A. Westerdaal, and P. J. Capel. 1991. A single amino acid in the second Ig-like domain of the human Fc gamma receptor II is critical for human IgG2 binding. J.Immunol. 147:1338-1343.
 57. WHO. 1999. Malaria, 1982-97. Weekly Epidemiol Record 74:265-272.

Claims

1. An antigen based vaccine against malaria comprising a fusion protein derived from *Plasmodium falciparum* Glutamate-rich protein (GLURP) genetically coupled to at least one other *Plasmodium falciparum* derived protein or a homologue of said fusion protein.
2. An antigen based vaccine against malaria according to claim 1 where the protein genetically coupled to GLURP is derived from the Merozoite surface protein 3 (MSP3) from *Plasmodium falciparum*.
3. A vaccine according to claim 2 comprising SEQ ID NO 1
4. A vaccine according to claim 2 or 3 further comprising an immunogenic fragment of a protein derived from *Plasmodium falciparum*
5. A fusion protein comprising SEQ ID NO. 1 or a homologue hereof.
6. A fusion protein according to claim 5 further comprising one or more immunogenic fragments of one or more proteins derived from *Plasmodium falciparum*.
7. A fusion protein according to claim 6 where the immunogenic fragment is chosen from CS, MSP1, MSP2, MSP4, MSP5, MSP6, AMA1, Pf155/RESA, RAP1, EBA-175, pfEMP1, EXP1, LSA1, LSA3, Pf25, Pf45/48, Pf230, Pf27, Pf16, or Pf28.
8. Preparation of a fusion protein according to claim 5-7 from a recombinant *Lactococcus sp.*
9. A nucleic acid comprising SEQ ID NO. 2 or a homologue hereof.
10. A nucleic acid coding for a fusion protein according to claim 6 or 7
11. Use of a nucleic acid according to claim 9 or 10 for the preparation of a vaccine.

12. A vaccine comprising a recombinant BCG expressing the nucleic acid sequence according to claim 9 or 10.

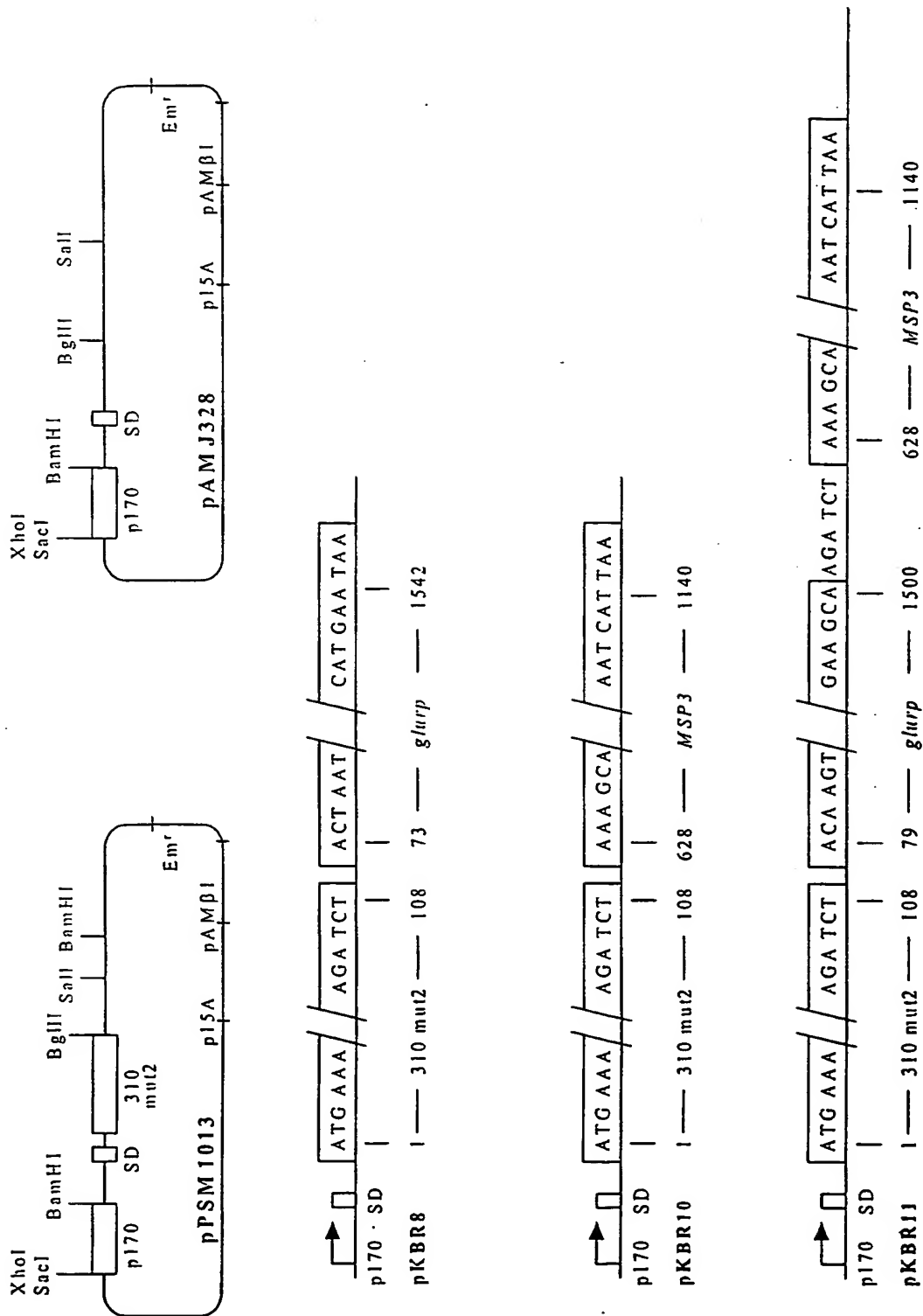


Figure 1

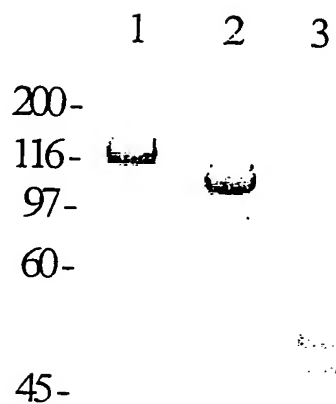
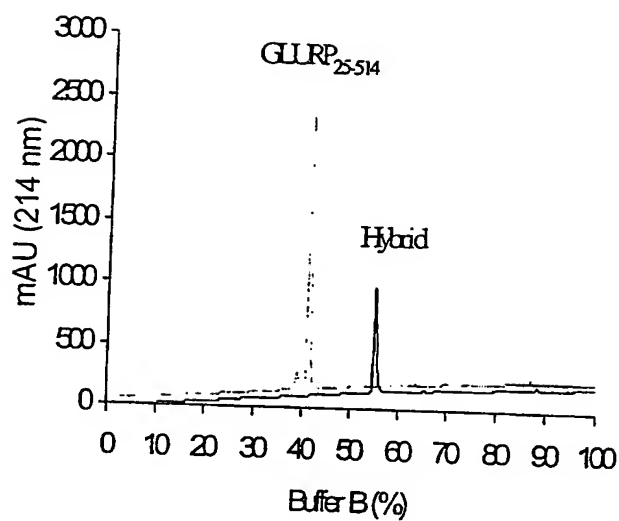
A**B**

Figure 2a

AERSTNTSENRRNKRIGGPKLRGNVTNISKFPDSNKGKIRGSNDKLNKNSEDLVLEQSEKSLVSENVPGLDIDDIPKESIFIQEDQEGQTHSELNPETSEH
SKDLNNGSKNESSDIISENKSNKVQHHFESLSDELLENSSQDNLDKDTISTEPFPNQKHKLQDLNDEPLEPFPPTQIHKOYKEKNLINEEDSEDPFPR
QKHKKVDNHNEEKNVFHENGSAANGQSLKLSFDEHLKDEKIENEPLVHENLSIPNDPIEQILNQPEQETNIQEQLYNEKQNVEEKQNSQIPSLDLKEPT
NEDILPNHNPLENIKQSESEINHVDHALPKENIDKLDNQKEHIDQSQHNINVLQENNINNHQLEPQEKPNIESFEPKNIDSEIILPENVETEEIIDDVP
SPKHSNHETFEETSESEHEEAUVSEKNAHETVEHEETVQSESNPEKADNDGNVSQNSNNELNENEFVESEKSEHEAAENEESSLEEGHHE

Figure 2b

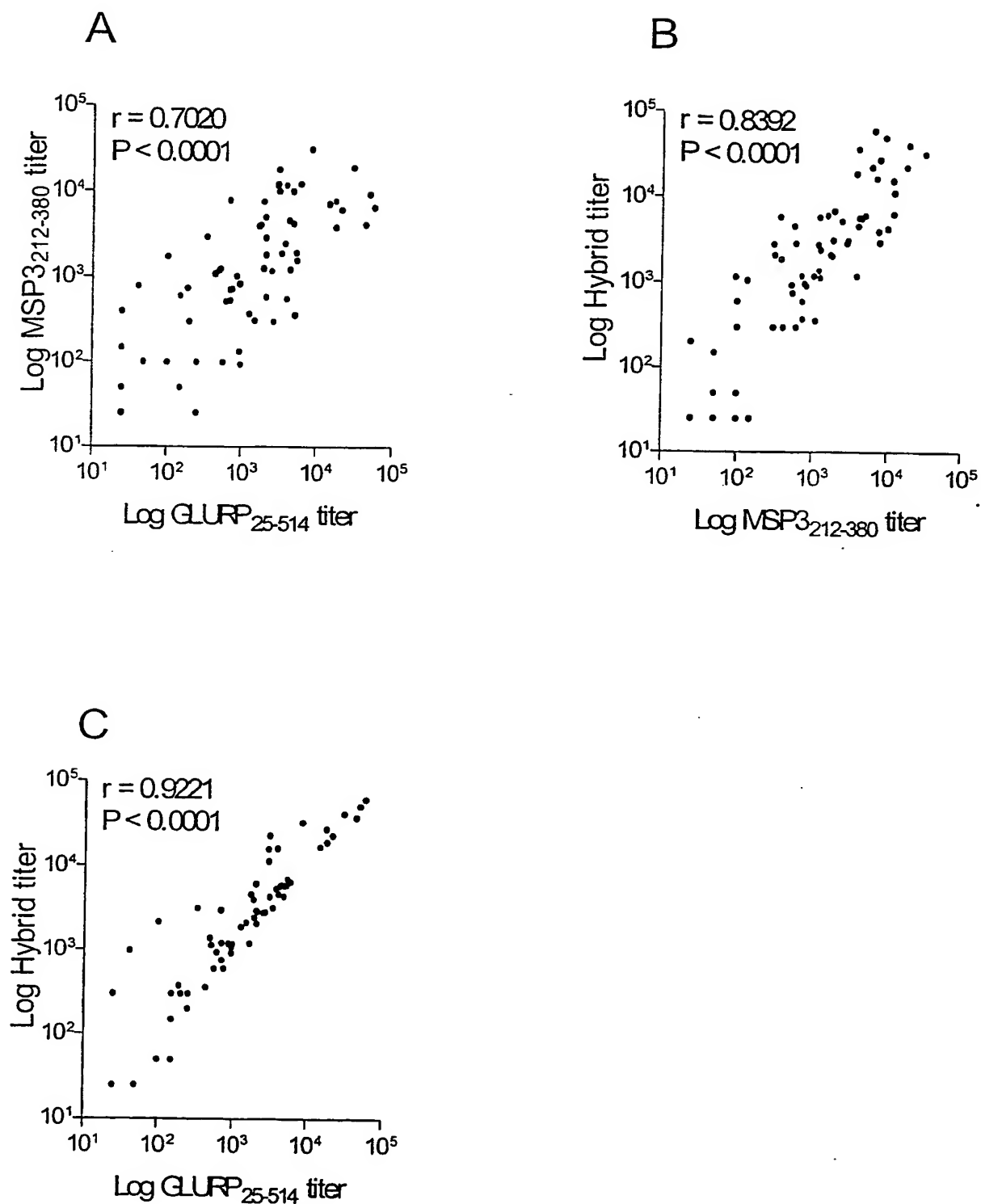


Figure 3

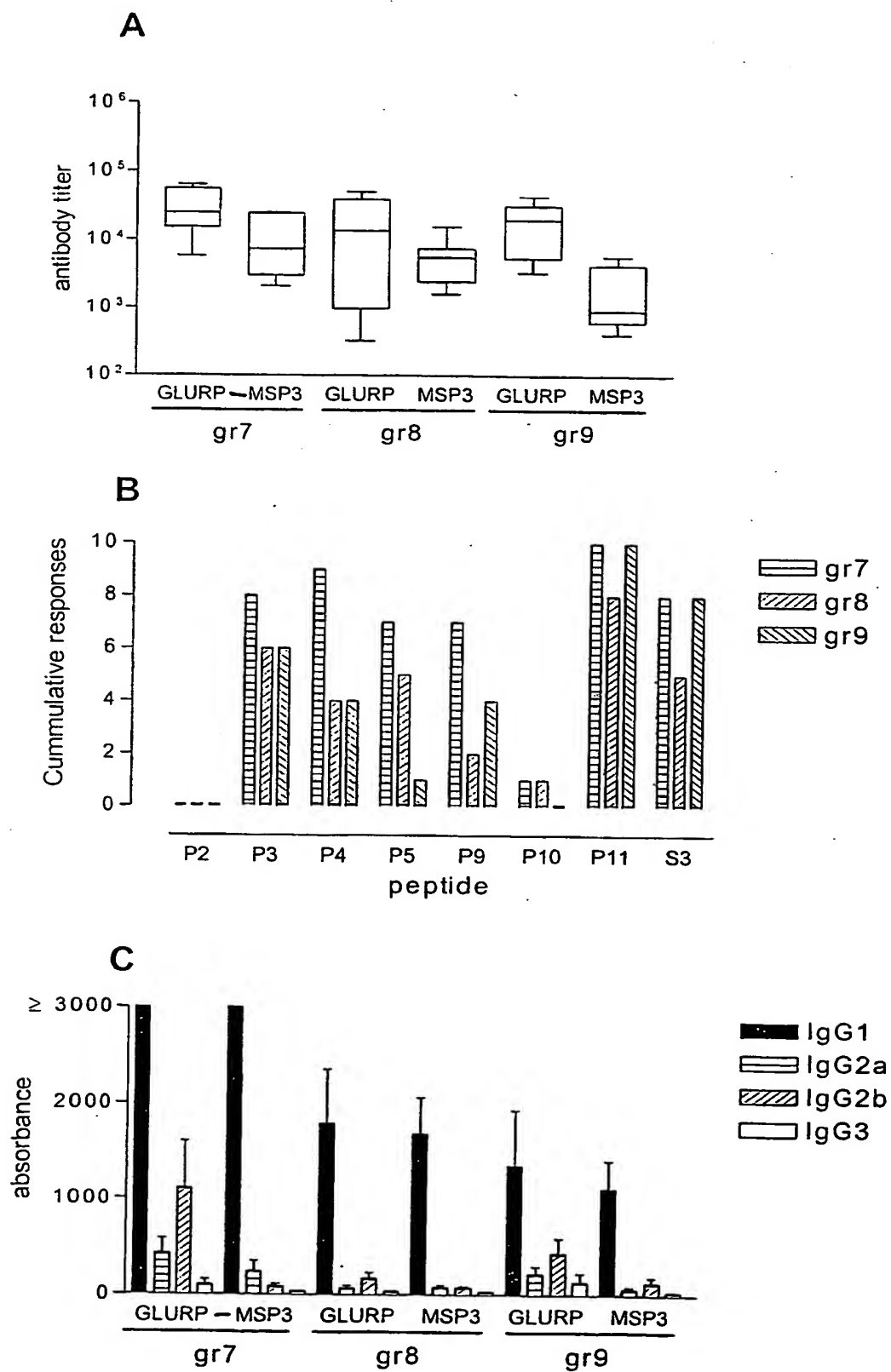


Figure 4
5/8

SUBSTITUTE SHEET (RULE 26)

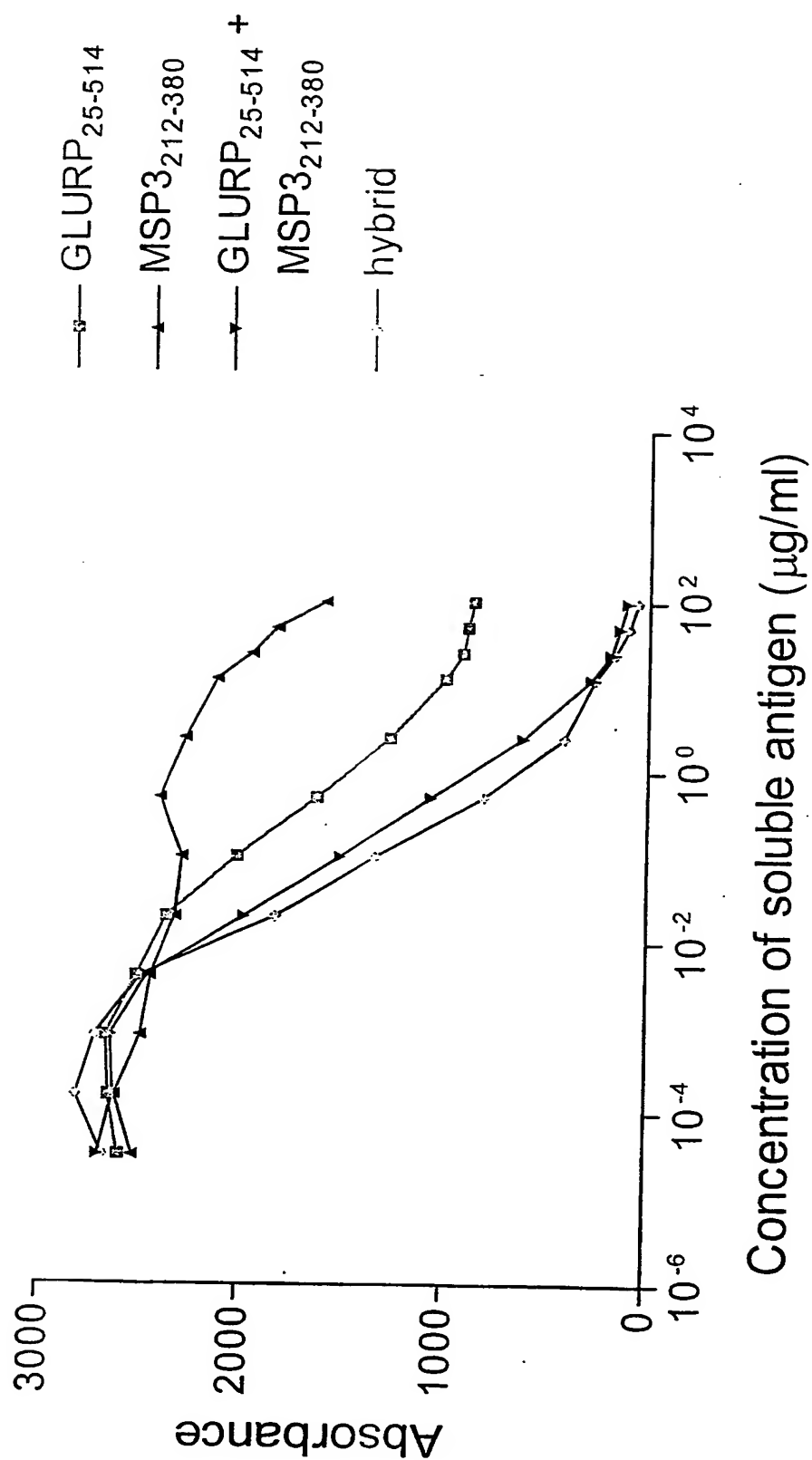


Figure 5

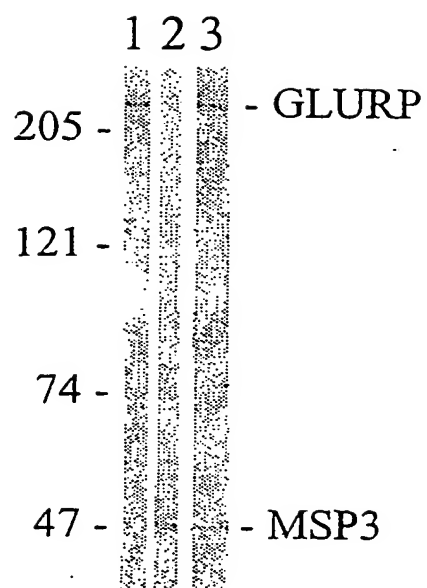


Figure 6

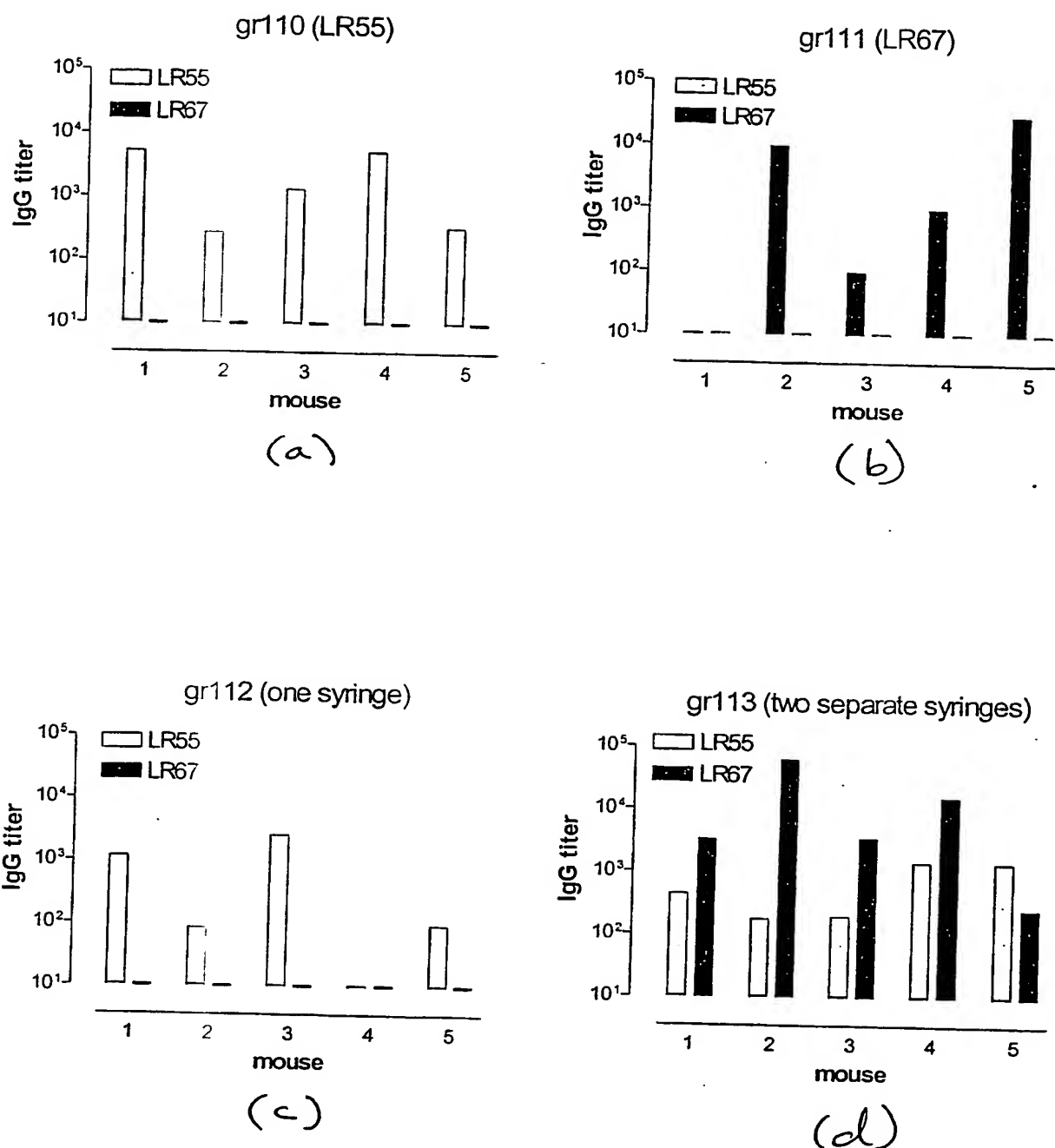


Figure 7

SEQUENCE LISTING

<110> Statens Serum Institut

<120> Vaccines comprising chimeric malaria proteins derived from Plasmodium falciparum

<130> 15007dk

<160> 2

<170> PatentIn version 3.1

<210> 1

<211> 651

<212> PRT

<213> Plasmodium falciparum

<400> 1

Ala Glu Arg Ser Thr Ser Glu Asn Arg Asn Lys Arg Ile Gly Gly Pro
 1 5 10 15

Lys Leu Arg Gly Asn Val Thr Ser Asn Ile Lys Leu Pro Ser Asn Asn
 20 25 30

Lys Gly Lys Ile Ile Arg Gly Ser Asn Asp Glu Leu Asn Lys Asn Ser
 35 40 45

Glu Asp Val Leu Glu Gln Ser Glu Lys Ser Leu Val Ser Glu Asn Val
 50 55 60

Pro Ser Gly Leu Asp Ile Asp Asp Ile Pro Lys Glu Ser Ile Phe Ile
 65 70 75 80

Gln Glu Asp Gln Glu Gly Gln Thr His Ser Glu Leu Asn Pro Glu Thr
 85 90 95

Ser Glu His Ser Lys Asp Leu Asn Asn Asn Asp Ser Lys Asn Glu Ser
 100 105 110

Ser Asp Ile Ile Ser Glu Asn Asn Lys Ser Asn Lys Val Gln Asn His
 115 120 125

Phe Glu Ser Leu Ser Asp Leu Glu Leu Leu Glu Asn Ser Ser Gln Asp
 130 135 140

Asn Leu Asp Lys Asp Thr Ile Ser Thr Glu Pro Phe Pro Asn Gln Lys
 145 150 155 160

His Lys Asp Leu Gln Gln Asp Leu Asn Asp Glu Pro Leu Glu Pro Phe
 165 170 175

Pro Thr Gln Ile His Lys Asp Tyr Lys Glu Lys Asn Leu Ile Asn Glu
 180 185 190

Glu Asp Ser Glu Pro Phe Pro Arg Gln Glu His Lys Lys Val Asp Asn
 195 200 205

His Asn Glu Glu Lys Asn Val Phe His Glu Asn Gly Ser Ala Asn Gly
 210 215 220

Asn Gln Gly Ser Leu Lys Leu Lys Ser Phe Asp Glu His Leu Lys Asp
 225 230 235 240

Glu Lys Ile Glu Asn Glu Pro Leu Val His Glu Asn Leu Ser Ile Pro
 245 250 255

Asn Asp Pro Ile Glu Gln Ile Leu Asn Gln Pro Glu Gln Glu Thr Asn
 260 265 270

Ile Gln Glu Gln Leu Tyr Asn Glu Lys Gln Asn Val Glu Glu Lys Gln
 275 280 285

Asn Ser Gln Ile Pro Ser Leu Asp Leu Lys Glu Pro Thr Asn Glu Asp
 290 295 300

Ile Leu Pro Asn His Asn Pro Leu Glu Asn Ile Lys Gln Ser Glu Ser
 305 310 315 320

Glu Ile Asn His Val Gln Asp His Ala Leu Pro Lys Glu Asn Ile Ile
 325 330 335

Asp Lys Leu Asp Asn Gln Lys Glu His Ile Asp Gln Ser Gln His Asn
 340 345 350

Ile Asn Val Leu Gln Glu Asn Asn Ile Asn Asn His Gln Leu Glu Pro
 355 360 365

Gln Glu Lys Pro Asn Ile Glu Ser Phe Glu Pro Lys Asn Ile Asp Ser
 370 375 380

Glu Ile Ile Leu Pro Glu Asn Val Glu Thr Glu Glu Ile Ile Asp Asp
 385 390 395 400

Val Pro Ser Pro Lys His Ser Asn His Glu Thr Phe Glu Glu Glu Thr
 405 410 415
 Ser Glu Ser Glu His Glu Glu Ala Val Ser Glu Lys Asn Ala His Glu
 420 425 430
 Thr Val Glu His Glu Glu Thr Val Ser Gln Glu Ser Asn Pro Glu Lys
 435 440 445
 Ala Asp Asn Asp Gly Asn Val Ser Gln Asn Ser Asn Asn Glu Leu Asn
 450 455 460
 Glu Asn Glu Phe Val Glu Ser Glu Lys Ser Glu His Glu Ala Arg Ser
 465 470 475 480
 Lys Ala Lys Glu Ala Ser Ser Tyr Asp Tyr Ile Leu Gly Trp Glu Phe
 485 490 495
 Gly Gly Gly Val Pro Glu His Lys Lys Glu Glu Asn Met Leu Ser His
 500 505 510
 Leu Tyr Val Ser Ser Lys Asp Lys Glu Asn Ile Ser Lys Glu Asn Asp
 515 520 525
 Asp Val Leu Asp Glu Lys Glu Glu Glu Ala Glu Glu Thr Glu Glu Glu
 530 535 540
 Glu Leu Glu Glu Lys Asn Glu Glu Glu Thr Glu Ser Glu Ile Ser Glu
 545 550 555 560
 Asp Glu Glu Glu Glu Glu Glu Glu Lys Glu Glu Glu Asn Glu Lys
 565 570 575
 Lys Lys Glu Gln Glu Lys Glu Gln Ser Asn Glu Asn Asn Asp Gln Lys
 580 585 590
 Lys Asp Met Glu Ala Gln Asn Leu Ile Ser Lys Asn Gln Asn Asn Asn
 595 600 605
 Glu Lys Asn Val Lys Glu Ala Ala Glu Ser Ile Met Lys Thr Leu Ala
 610 615 620
 Gly Leu Ile Lys Gly Asn Asn Gln Ile Asp Ser Thr Leu Lys Asp Leu

625

630

635

640

Val Glu Glu Leu Ser Lys Tyr Phe Lys Asn His
 645 650

<210> 2
 <211> 1953
 <212> DNA
 <213> Plasmodium falciparum

<400> 2
 gccgaaagat ctacaagtga gaatagaaat aaacgaatcg ggggtcctaa attaaggggt 60
 aatgttacaa gtaatatataa gctgccatca aataacaaag gtaaaattat aagagggttcg 120
 aatgatgaac ttaataaaaa ctctgaagat gttttagaac aaagcgaaaa atcgcttggt 180
 tcagaaaatg ttcttagtgg attagatata gatgatatcc ctaaagaatc tatttttatt 240
 caagaagatc aagaaggtca aactcattct gaattaaatc ctgaaacatc agaacatagt 300
 aaagatttaa ataataatga ttcaaaaaat gaatctagtg atattatttc agaaaataat 360
 aaatcaaata aagtacaaaa tcattttgaa tcattatcag atttagaatt acttgaaaat 420
 tcttcacaag ataatttaga caaagatata atttcaacag aaccttttcc taatcaaaaa 480
 cataaagact tacaacaaga tttaaatgat gaaccttttag aaccttttcc tacacaaata 540
 cataaagatt ataaagaaaa aaatttaata aatgaagaag attcagaacc atttcccaga 600
 caagagcata aaaaggtaga caatcataat gaagaaaaaa acgtatttca tgaaaatggg 660
 tctgcaaagtg gtaatcaagg aagtttgaaa cttaaatacat tcgatgaaca tttaaagat 720
 gaaaaaatag aaatgaacc acttggttcat gaaaatttat ccataccaaa tgatccaata 780
 gaacaaatat taaatcaacc tgaacaagaa acaaatatcc aggaacaatt gtataatgaa 840
 aaacaaaatg ttgaagaaaa acaaaattct caaatacctt cgttagattt aaaagaacca 900
 acaaatgaag atattttacc aaatcataat ccattagaaa atataaaaca aagtgaatca 960
 gaaataaatc atgtacaaga tcatgcgcta ccaaagaga atataataga caaacttgat 1020
 aatcaaaaag aacacatcga tcaatcacia cataatataa atgtattaca agaaaataac 1080
 ataaacaatc accaattaga acctcaagag aaacctataa ttgaatcggt tgaacctaaa 1140
 aatatagatt cagaaattat tcttctgaa aatggtgaaa cagaagaaat aatagatgat 1200
 gtgccttccc ctaaacattc taaccatgaa acatttgaag aagaaacaag tgaatctgaa 1260
 catgaagaag ccgtatctga aaaaaatgcc caggaactg tcgaacatga agaaactgtg 1320
 tctcaagaaa gcaatcctga aaaagctgat aatgatggaa atgtatctca aaacagcaac 1380

aacgaattaa atgaaaatga attcgttgaa tcggaaaaaa gcgagcatga agcaagatct	1440
aaagcaaaaag aagcttctag ttatgattat attttaggtt gggaatttgg aggaggcggt	1500
ccagaacaca aaaaagaaga aaatatgtta tcacatttat atgtttcctc aaaggataag	1560
gaaaatatat ctaaggaaaa tgatgatgta ttagatgaga aggaagaaga ggcagaagaa	1620
acagaagaag aagaacttga agaaaaaat gaagaagaaa cagaatcaga aataagtga	1680
gatgaagaag aagaagaaga agaagaaaag gaagaagaaa atgaaaaaa aaaagaacaa	1740
gaaaaagaac aaagtaatga gaataatgat caaaaaaag atatggaagc acagaattta	1800
atttctaaaa accagaataa taatgagaaa aacgtaaaag aagctgctga aagcatcatg	1860
aaaacttttag ctggtttaat caagggaat aatcaaatag attctacctt aaaagattta	1920
gtagaagaat tatccaaata ttttaaaaat cat	1953

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 03/00759

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/015 C12N15/30 A61P33/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CARVALHO, L. J. M. ET AL: "Malaria Vaccine: Candidate Antigens, Mechanisms, Constraints and Prospects" SCAND. J. IMMUNOL., vol. 56, 2002, pages 327-343, XP002273865	1
Y	see table 2 (MSP-3 and GLURP), and page 336-37 (Multistage approaches)	2-12
Y	--- WO 90 02811 A (STATENS SERUMINSTITUT) 22 March 1990 (1990-03-22) the whole document --- -/--	1-12

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

18 March 2004

Date of mailing of the international search report

- 7. 04 2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

MICAEL OWALD /EÖ

INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK 03/00759

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>OEUVRAY, CLAUDE ET AL: "Cytophilic Immunoglobulin Responses to Plasmodium falciparum Glutamate-Rich Protein Are Correlated with Protection against Clinical Malaria in Dielmo, Senegal" INFECTION AND IMMUNITY, vol. 68, no. 5, May 2000 (2000-05), pages 2617-2620, XP002273866 the whole document</p> <p>---</p>	1-12
Y	<p>OEUVRAY, C ET AL: "Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes" BLOOD, vol. 84, no. 5, 1994, pages 1594-1602, XP002273867 the whole document</p> <p>---</p>	1-12
Y	<p>CARVALHO, L. J. M. ET AL: "IM-95-Immunization of saimiri sciureus monkeys with MSP-3 and glurp, two plasmodium falciparum antigens targets of protective antibodies" MEMORIAS DO INSTITUTO OSWALDO CRUZ, vol. 94, no. 11, November 1999 (1999-11), page 216 XP002273868 the whole document</p> <p>---</p>	1-12
Y	<p>WO 00 50077 A (SMITHKLINE BEECHAM BIOLOG ;VERRIEST CHRISTOPHE (BE); LOBET YVES (B) 31 August 2000 (2000-08-31) page 10, line 22 - line 28</p> <p>---</p>	1-12
A	<p>BREDMOSE, L. ET AL: "Development of a heterologous gene expression system for use in lactococcus lactis" RECOMBINANT PROTEIN PRODUCTION WITH PROKARYOTIC AND EUKARYOTIC CELLS, 2001, pages 269-275, XP002274030 the whole document</p> <p>-----</p>	1-12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK 03/00759

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9002811	A	22-03-1990	AU 645482 B2	20-01-1994
			AU 4335089 A	02-04-1990
			WO 9002811 A1	22-03-1990
			EP 0434751 A1	03-07-1991
			JP 4501661 T	26-03-1992
			US 5231168 A	27-07-1993

WO 0050077	A	31-08-2000	AU 2672700 A	14-09-2000
			AU 3158900 A	14-09-2000
			BR 0008963 A	27-11-2001
			CA 2363118 A1	31-08-2000
			CA 2363637 A1	31-08-2000
			CN 1348466 T	08-05-2002
			CZ 20013082 A3	13-02-2002
			WO 0050460 A1	31-08-2000
			WO 0050077 A1	31-08-2000
			EP 1155037 A1	21-11-2001
			EP 1156825 A2	28-11-2001
			HU 0200049 A2	29-05-2002
			JP 2002537354 A	05-11-2002
			NO 20014130 A	13-09-2001
			NZ 513679 A	28-09-2001
			PL 350993 A1	24-02-2003
			TR 200102506 T2	21-06-2002
			US 2003170229 A1	11-09-2003
			ZA 200107016 A	17-01-2003
			AU 3281100 A	14-09-2000
			BR 0008964 A	27-11-2001
			CA 2363641 A1	31-08-2000
			CZ 20013081 A3	13-02-2002
			WO 0050461 A1	31-08-2000
			EP 1155038 A1	21-11-2001
			HU 0105490 A2	29-04-2002
			JP 2002537403 A	05-11-2002
			NO 20014131 A	02-10-2001
			NZ 513680 A	28-09-2001
			PL 350992 A1	24-02-2003
			TR 200102493 T2	21-02-2002
			ZA 200107015 A	26-11-2002

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)